FORM PCT/DO/EO/00 (REV. 10/00)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
1599-0206P	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
09/937215 NEW	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/KR99/00131	March 24, 1999	NONE

TITLE OF INVENTION
TREHALOSE SYNTHASE PROTEIN, GENE, PLASMIDS, MICROORGANISMS, AND A PROCESS FOR PRODUCING TREHALOSE

APPLICANT(S) FOR DO/EO/US
LEE, Se Yong; SONG, Eun Kyung; PARK, Yearn Hung; KWON, Sang Ho; LEE, Kwang Ho; KIM, Chang Gyeom; LEE, Jin Ho; CHUNG, Sung Oh; JEON, Yeong Joong

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau). WO 00/56868
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is transmitted herewith.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). (Photocopy)
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98, Form PTO-1449(s), and International Search Report (PCT/ISA/210) with 0 document(s).
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:
    1. International Preliminary Examination Report (PCT/IPEA/409)
    2. PCT Request (PCT/RO/101)
    3. Sequence Listing (6 pages)
    4. Six (6) sheets of formal drawings
    5. Receipt concerning Deposit of Microorganisims

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/NEW 37215	PCT/KR99/00131	1599-0206P

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1,000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1,000.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	9 - 20 =	0	X **$18.00**	$ 0	
Independent Claims	3 - 3 =	0	X **$80.00**	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$ 0	
			TOTAL OF ABOVE CALCULATIONS =	$ 1,000.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$	
			SUBTOTAL =	$ 1,000.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
			TOTAL NATIONAL FEE =	$ 1,000.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00	
			TOTAL FEES ENCLOSED =	$ $1,040.00	
			Amount to be: refunded	$	
			charged	$	

a. ☒ A check in the amount of $ **1,040.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Send all correspondence to:
Birch, Stewart, Kolasch & Birch, LLP or Customer No. 2292
P.O. Box 747
Falls Church, VA 22040-0747
(703)205-8000

Date: September 24, 2001

By Reg No 32,334
Joseph A. Kolasch, #22,463

/kw

PATENT
1599-0206P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: LEE, Se Yong et al.

Int'l. Appl. No.: PCT/KR99/00131

Appl. No.: NEW Group:

Filed: September 24, 2001 Examiner:

For: TREHALOSE SYNTHASE PROTEIN, GENE, PLASMIDS, MICROORGANISMS, AND A PROCESS FOR PRODUCING TREHALOSE

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

September 24, 2001

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please insert the following paragraph before the paragraph beginning on page 1, line 1:

--This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/KR99/00131 which has an International filing date of March 24, 1999, which designated the United States of America and was published in English.- -

Please replace the paragraph beginning on page 13, line 13 with the following rewritten paragraph:

--The enzymatic reaction is conducted at pH 6.0 to 11, preferably pH 7.0 to 10, and at temperatures of 4°C to 45°C, preferably 20°C to 40°C. Maltose can be used as a substrate in a concentration of less than 50%. The trehalose synthase enzyme can be used in a pure form or in crushed cells.--

Please replace the paragraph beginning on page 16, line 19, with the following rewritten paragraph:

--The pure chromosomal DNAs isolated from *Pseudomonas stutzeri* were partially digested with restriction enzyme *Sau*3AI at 37°C for 15 to 30 minutes. The restriction enzyme was inactivated with heat and agarose gel electrophoresis was carried out to obtain 3 to 10 kb DNA fragments. As shown in Figure 5, plasmid pUC18 was digested with *Bam*HI and was treated with calf intestinal phosphatase. The cleaved DNAs were mixed with 3 to 10 kb DNA fragments previously obtained and ligation with T4 DNA ligase was allowed at 15°C for 16 hours. The recombinants thus obtained were used for transofrmation. The transformation was carried out by electroporation as follows. *E coli* NM522 was cultured on LB medium for 14 to 15 hours. The resulting culture was inoculated on 1L LB so that initial absorbency became 0.07 to 0.1 at 600 nm, and then cultivation was allowed until the absorbency reached 0.8. The cells were centrifuged and suspended in 1L of HEPES [N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic

acid)] buffer solution. The cells were again centrifuged and suspended in 500 ml of cold sterile deionized distilled water. The cells were again centrifuged and suspended in 20 ml of 10% glycerol solution. The cells were again centrifuged and suspended in 2 to 3 ml of 10% glycerol solution so that the cell concentration was adjusted to 2-4 x 10^{10}/ml. The cell suspension was rapidly frozen and stored at -70°C. The frozen cells could be used for about one month during which time their transformation frequency did not decrease. 40μL of frozen cell suspension was thawed in ice and the restored suspension was mixed with the ligated DNA solution. The mixture was put in a gene pulser cuvette with a diameter of 0.2 cm and the capacitance and strength of electric field was fixed at 25 uF and 12.5 kV/cm, respectively. After a single electric pulse was passed at resistance of 200 to 400 Ω, 1 ml of SOC medium was immediately added and cultured at 37°C for 1 hour. The culture was streaked on LB-ampicillin agar medium and cultivation was allowed for 24 hours to obtain at least fifty thousand colonies. These colonies were together cultured in LB broth for 2 hours. DNA was purely isolated using an alkaline lysis and the genomic library was constructed therefrom.--

Please replace the paragraph beginning on page 18, line 22, with the following rewritten paragraph:

--The plasmid pCJ104 was subjected to single, double, and triple-digest procedures using about twenty restriction enzymes,

such as *Aat*II, *Bam*HI, *Bgl*II, *Sma*I, *Eco*RI, *Eco*RV, *Kpn*I, *Nco*I, *Nde*I, *Pst*I, *Sac*I, *Sac*II, *Sal*I, *Sph*I and *Xho*I. DNA fragments were analyzed by electrophoresis through agarose gel and compared to construct the restriction map.--

Please replace the table beginning on page 18, line 5, with the following rewritten table:

--Table 3. Enzyme Titration

Microorganisms	Specific activity of enzyme (U*/mg of protein)	Culture Titer (U/ml of culture solution)
Pseudomonas stutzeri CJ38	0.1	0.023
E. coli ATCC35467/pUC18	0	0
E. coli ATCC35467/pCJ104	0.26	0.175

*U-μmol trehalose/minutes--

Please replace the heading beginning on page 18, line 28, with the following rewritten heading:

--**Example 8**--

Please replace the table beginning on page 20, line 17, with the following rewritten table:

--Table 5

Microorganisms	Specific activity of enzyme (U/mg of protein)	Culture Titer of 5 L Fermenter (U/ml of culture)
E coli ATCC35467/pCJ121	0.43	-
E. coli ATCC35467/pCJ122	4.95	30
E. coli ATCC35467/pCJ123	0	-

--

In the Claims:

Please amend the claims as follows:

3. (Amended) A recombinant plasmid containing the trehalose synthase gene of claim 2.

4. (Amended) The recombinant plasmid according to claim 3 which is recombinant plasmid pCJ122.

5. (Amended) A transformed E. coli with the recombinant plasmid of claim 3.

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application.

The claims have been amended to correct the dependency thereof.

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly solicited.

Attached hereto is a marked-up copy of the changes made to the application by this Amendment.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By Joe McMurry Reg No. 32,334
for Joseph A. Kolasch, #22,463

P.O. Box 747

JAK/kw

Falls Church, VA 22040-0747

1599-0206P

(703) 205-8000

Attachment: VERSION WITH MARKINGS TO SHOW CHANGES MADE

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

A paragraph has been added before the paragraph beginning on page 1, line 1.

The paragraph beginning on page 13, line 13 has been amended as follows:

--The enzymatic reaction is conducted at pH 6.0 to [7.0] 11, preferably pH 7.0 to 10, and at temperatures of 4°C to 45°C, preferably 20°C to 40°C. Maltose can be used as a substrate in a concentration of less than 50%. The trehalose synthase enzyme can be used in a pure form or in crushed cells.--

The paragraph beginning on page 16, line 19, has been amended as follows:

--The pure chromosomal DNAs isolated from *Pseudomonas stutzeri* were partially digested with restriction enzyme *Sau*3AI at 37°C for 15 to 30 minutes. The restriction enzyme was inactivated with heat and agarose gel electrophoresis was carried out to obtain 3 to 10 kb DNA fragments. As shown in Figure 5, plasmid pUC18 was digested with *Bam*HI and was treated with calf intestinal phosphatase. The cleaved DNAs were mixed with 3 to 10 kb DNA fragments previously obtained and ligation with T4 DNA ligase was allowed at 15°C for 16 hours. The recombinants thus obtained were used for transofrmation. The transformation was

carried out by electroporation as follows. *E coli* NM522 was cultured on LB medium for 14 to 15 hours. The resulting culture was inoculated on 1L LB so that initial absorbency became 0.07 to 0.1 at 600 nm, and then cultivation was allowed until the absorbency reached 0.8. The cells were centrifuged and suspended in 1L of HEPES [N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)] buffer solution. The cells were again centrifuged and suspended in 500 ml of cold sterile deionized distilled water. The cells were again centrifuged and suspended in 20 ml of 10% glycerol solution. The cells were again centrifuged and suspended in 2 to 3 ml of 10% glycerol solution so that the cell concentration was adjusted to 2-4 x [1,010]10^{10}/ml. The cell suspension was rapidly frozen and stored at -70°C. The frozen cells could be used for about one month during which time their transformation frequency did not decrease. 40μL of frozen cell suspension was thawed in ice and the restored suspension was mixed with the ligated DNA solution. The mixture was put in a gene pulser cuvette with a diameter of 0.2 cm and the capacitance and strength of electric field was fixed at 25 uF and 12.5 kV/cm, respectively. After a single electric pulse was passed at resistance of 200 to 400 Ω, 1 ml of SOC medium was immediately added and cultured at 37°C for 1 hour. The culture was streaked on LB-ampicillin agar medium and cultivation was allowed for 24 hours to obtain at least fifty thousand colonies. These colonies were together cultured in LB broth for 2 hours. DNA was purely

isolated using an alkaline lysis and the genomic library was constructed therefrom.--

The table beginning on page 18, line 5, has been amended as follows:

--Table 3. Enzyme Titration

Microorganisms	[Non-enzymatic activities] Specific activity of enzyme (U*/mg of protein)	Culture Titer (U/ml of culture solution)
Pseudomonas stutzeri CJ38	0.1	0.023
E. coli ATCC35467/pUC18	0	0
E. coli ATCC35467/pCJ104	0.26	0.175

*U-μmol trehalose/minutes--

The paragraph beginning on page 18, line 22, has been amended as follows:

--The plasmid pCJ104 was subjected to single, double, and triple-digest procedures using about twenty restriction enzymes, such as *Aat*II, *Bam*HI, *Bgl*II, *Sma*I, *Eco*RI, *Eco*RV, *Kpn*I, *Nco*I, *Nde*I, *Pst*I, *Sac*I, *Sac*II, *Sal*I, *Sph*I and *Xho*I. DNA fragments were analyzed by electrophoresis through agarose gel and compared to construct the restriction map.--

The heading beginning on page 18, line 28, has been amended as follows:

--**Example [3]8**--

The table beginning on page 20, line 17, has been amended as follows:

--Table 5

Microorganisms	[Non-enzymatic activities] Specific activity of enzyme (U/mg of protein)	Culture Titer of 5 L Fermenter (U/ml of culture)
E coli ATCC35467/pCJ121	0.43	-
E. coli ATCC35467/pCJ122	4.95	30
E. coli ATCC35467/pCJ123	0	-

--

In the Claims:

The claims have been amended as follows:

3. (Amended) A recombinant plasmid containing the trehalose synthase gene of claim [1] 2.

4. (Amended) The recombinant plasmid according to claim [1] 3 which is recombinant plasmid pCJ122.

5. (Amended) A transformed E. coli with the recombinant plasmid of claim [1] 3.

BOX SEQUENCE
PATENT
1599-0206P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	Lee, Se Yong et al.	Conf.:	6757
Appl. No.:	09/937,215	Group:	UNASSIGNED
Filed:	September 24, 2001	Examiner:	UNASSIGNED

For: TREHALOSE SYNTHASE PROTEIN, GENE, PLASMIDS, MICROORGANISMS, AND A PROCESS FOR PRODUCING TREHALOSE

AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

February 6, 2002

Sir:

In response to the Notification of Missing Requirements mailed December 6, 2001, the following amendments and remarks are respectfully submitted in connection with the above-identified application.

In the Specification:

Please replace Table 1 beginning on page 11, line 14, with the following amended Table 1:

--

Table 1. N-terminal Sequences of Trehalose Synthase Proteins

Source of Trehalose Synthase		N-terminal Sequence
Known Microbes	*Thermus aquaticus* ATCC 33923	M-D-P-L-W-Y-K-D-A-V-I-Y-Q- (SEQ ID NO: 3)
	Pimelobacter sp. R48	S-T-V-L-G-E-E-P-E-W- F-R-T-A-V-F-Y-E- (SEQ ID NO: 4)
	Pseudomonas putida H262	G-K-W-P-R-P-A-A-F-I-D- (SEQ ID NO: 5)
Transformed *E. coli* of the Present Invention		S-I-P-D-N-T-Y-I-E-W-L-V- (SEQ ID NO: 6)

--

Please delete the Sequence Listing of record. Please insert the Substitute Sequence Listing enclosed herewith immediately after the abstract.

REMARKS

Enclosed herewith in full compliance with 37 C.F.R. §§1.821-1.825 is a Substitute Sequence Listing to be inserted into the specification as indicated above. The Substitute Sequence Listing in no way introduces new matter into the specification. Also submitted herewith in full compliance with 37 C.F.R. §§1.821-1.825 is a disk copy of the Substitute Sequence Listing. The disk copy

of the Sequence Listing, file "1599-0206P.ST25", is identical to the paper copy, except that it lacks formatting.

The amendments made to the Specification are intended to reference each amino acid sequence by a unique SEQ ID NO. No new matter is introduced by these amendments.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____
Joseph A. Kolasch, #22,463

P.O. Box 747
Falls Church, VA 22040-0747
(703) 205-8000

JAK/BCF
1599-0206P

Attachments:
Disk Copy of Sequence Listing
Paper Copy of Sequence Listing
Copy of Notice to Comply
Version with markings

VERSION WITH MARKINGS TO SHOW CHANGES MADE

(Material being **added** is shown as bold and underlined. No material is being deleted)

Table 1 beginning on page 11, line 14:

--

Source of Trehalose Synthase		N-terminal Sequence
Known Microbes	*Thermus aquaticus* ATCC 33923	M-D-P-L-W-Y-K-D-A-V-I-Y-Q- **(SEQ ID NO: 3)**
	Pimelobacter sp. R48	S-T-V-L-G-E-E-P-E-W- F-R-T-A-V-F-Y-E- **(SEQ ID NO: 4)**
	Pseudomonas putida H262	G-K-W-P-R-P-A-A-F-I-D- **(SEQ ID NO: 5)**
Transformed *E. coli* of the Present Invention		S-I-P-D-N-T-Y-I-E-W-L-V- **(SEQ ID NO: 6)**

--

TREHALOSE SYNTHASE PROTEIN, GENE, PLASMIDS, MICROORGANISMS, AND A PROCESS FOR PRODUCING TREHALOSE

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a trehalose-producing microorganism and a process for producing trehalose. It also relates to a novel trehalose synthase protein, a trehalose synthase gene, recombinant plasmids carrying said trehalose synthase gene, and transformed microorganisms with said recombinant plasmids.

DESCRIPTION OF THE PRIOR ART

Trehalose is a non-reducing disaccharide, two saccharides of which are linked by α-1,1 bond: α-D-glucopyranosyl-α-D-glucopyranoside. It has wide application in medicines, foods, and cosmetics. However, its utilization has been greatly restricted because its production to date has been inefficient and expensive.

Japanese Laid-open Patent Nos. Hei5-91890 and Hei6-145186 disclose methods for extracting trehalose from yeasts. There are several methods for isolating trehalose from fermented microorganism cultures, such as *Arthrobacter* (T. Suzuki, Agric. Biol. Chem., 33(2), 1969), *Nocardia* (Japanese Laid-open Patent No. Sho50-154485), *Micrococcus* (Japanese Laid-open Patent No. Hei6-319578), amino acid-fermenting yeast, *Brevibacterium* (Japanese Laid-open Patent No. Hei5-211882), and yeast (Yoshikwa, etc., Biosci. Biotech. Biochem., 1994, 58, 1226-12300). Additionally, a method for producing trehalose by using recombinant plants including bacterial genes capable of converting glucose into trehalose is described in M. Scher, Food Processing, April, 95-96, 1993. Japanese Laid-open Patent No. 83-216695 discloses a method for converting maltose into trehalose by using maltose phosphorylase and trehalose phosphorylase. However, these methods are not effective. because their procedures are complicated and their yields are low.

Several enzymatic methods have been published recently. Japanese Laid-open Patent No. Hei7-143876 and EPO 628630 A2 discloses a two-step enzymatic conversion method in which starch is converted into trehalose by maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase. Japanses Laid-open Patent No. Hei7-170977 and Korean Laid-open Patent No. 95-3444 disclose one-step enzymatic conversion methods in which maltose is directly converted into trehalose by trehalose synthase. However, there is still a need to increase the titer of the trehalose synthase enzyme so that production of trehalose from maltose becomes more efficient in yield and cost.

We have invested much effort over the last several years in isolating microorganisms able to convert maltose into trehalose from soil. We have successfully screened a novel strain which highly expresses trehalose and, unexpectedly, generates no byproducts, unlike all known microorganisms. Its morphological and physiological characteristics identify it as a novel *Pseudomonas stutzeri* strain. This strain has been designated as *Pseudomonas stutzeri* CJ38.

We isolated a trehalose synthase gene from chromosomes of *Pseudomonas stutzeri* CJ38 and determined its nucleotide sequence by cloning it into known vector pUC18 with restriction enzyme *Sau*3AI. In addition, we isolated a trehalose synthase protein from *Pseudomonas stutzeri* CJ38 and determined its amino acid sequence using standard methods. It was found that these sequences are apparently different from the sequences of the trehalose synthase gene and all proteins known hitherto. This invention was achieved by constructing recombinant plasmids carrying the trehalose synthase gene so that the trehalose synthase enzyme encoded in said gene can be expressed in large amounts.

SUMMARY OF THE INVENTION

The present invention provides a novel microorganism, *Pseudomonas stutzeri* CJ38, that produces trehalose from maltose. This strain was deposited at the Korea Culture Center of Microorganisms, Seoul, Korea, as the accession number KCCM 10150 on February 12, 1999 under the Budapest Treaty. This strain is very valuable

as it does not generate byproducts such as glucose when converts maltose into trehalose.

The present invention also provides a novel trehalose synthase protein with the following amino acid sequence:

Met Ser Ile Pro Asp Asn Thr Tyr Ile Glu Trp Leu Val Ser Gln
5 10 15
Ser Met Leu His Ala Ala Arg Glu Arg Ser Arg His Tyr Ala Gly
20 25 30
Gln Ala Arg Leu Trp Gln Arg Pro Try Ala Gln Ala Arg Pro Arg
35 40 45
Asp Ala Ser Ala Ile Ala Ser Val Trp Phe Thr Ala Tyr Pro Ala
50 55 60
Ala Ile Ile Thr Pro Glu Gly Gly Thr Val Leu Glu Ala Leu Gly
65 70 75
Asp Asp Arg Leu Trp Ser Ala Leu Ser Glu Leu Gly Val Gln Gly
80 85 90
Ile His Asn Gly Pro Met Lys Arg Ser Gly Gly Leu Arg Gly Arg
95 100 105
Glu Phe Thr Pro Thr Ile Asp Gly Asn Phe Asp Arg Ile Ser Phe
110 115 120
Asp Ile Asp Pro Ser Leu Gly Thr Glu Glu Gln Met Leu Gln Leu
125 130 135
Ser Arg Val Ala Ala Ala His Asn Ala Ile Val Ile Asp Asp Ile
140 145 150
Val Pro Ala His Thr Gly Lys Gly Ala Asp Phe Arg Leu Ala Glu
155 160 165
Met Ala Tyr Gly Asp Tyr Pro Gly Leu Tyr His Met Val Glu Ile
170 175 180
Arg Glu Glu Asp Trp Glu Leu Leu Pro Glu Val Pro Ala Gly Arg
185 190 195
Asp Ser Val Asn Leu Leu Pro Pro Val Val Asp Arg Leu Lys Glu
200 205 210

Lys His Tyr Ile Val Gly Gln Leu Gln Arg Val Ile Phe Phe Glu
215 220 225
Pro Gly Ile Lys Asp Thr Asp Trp Ser Val Thr Gly Glu Val Thr
230 235 240
Gly Val Asp Gly Lys Val Arg Arg Trp Val Tyr Leu His Tyr Phe
245 250 255
Lys Glu Gly Gln Pro Ser Leu Asn Trp Leu Asp Pro Thr Phe Ala
260 265 270
Ala Gln Gln Leu Ile Ile Gly Asp Ala Leu His Ala Ile Asp Val
275 280 285
Thr Gly Ala Arg Val Leu Arg Leu Asp Ala Asn Gly Phe Leu Gly
290 295 300
Val Glu Arg Arg Ala Glu Gly Thr Ala Trp Ser Glu Gly His Pro
305 310 315
Leu Ser Val Thr Gly Asn Gln Leu Leu Ala Gly Ala Ile Arg Lys
320 325 330
Ala Gly Gly Phe Ser Phe Gln Glu Leu Asn Leu Thr Ile Asp Asp
335 340 345
Ile Ala Ala Met Ser His Gly Gly Ala Asp Leu Ser Tyr Asp Phe
350 355 360
Ile Thr Arg Pro Ala Tyr His His Ala Leu Leu Thr Gly Asp Thr
365 370 375
Glu Phe Leu Arg Met Met Leu Arg Glu Val His Ala Phe Gly Ile
380 385 390
Asp Pro Ala Ser Leu Ile His Ala Leu Gln Asn His Asp Glu Leu
395 400 405
Thr Leu Glu Leu Val His Phe Trp Thr Leu His Ala Tyr Asp His
410 415 420
Tyr His Tyr Lys Gly Gln Thr Leu Pro Gly Gly His Leu Arg Glu
425 430 435
His Ile Arg Glu Glu Met Tyr Glu Arg Leu Thr Gly Glu His Ala
440 445 450

Pro Tyr Asn Leu Lys Phe Val Thr Asn Gly Val Ser Cys Thr Thr
455 460 465
Ala Ser Val Ile Ala Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala
470 475 480
Ile Gly Pro Ala Glu Val Glu Gln Ile Gln Arg Leu His Ile Leu
485 490 495
Leu Val Met Phe Asn Ala Met Gln Pro Gly Val Phe Ala Leu Ser
500 505 510
Gly Trp Asp Leu Val Gly Ala Leu Pro Leu Ala Pro Glu Gln Val
515 520 525
Glu His Leu Met Gly Asp Gly Asp Thr Arg Trp Ile Asn Arg Gly
530 535 540
Gly Tyr Asp Leu Ala Asp Leu Ala Pro Glu Ala Ser Val Ser Ala
545 550 555
Glu Gly Leu Pro Lys Ala Arg Ser Leu Tyr Gly Ser Leu Ala Glu
560 565 570
Gln Leu Gln Arg Pro Gly Ser Phe Ala Cys Gln Leu Lys Arg Ile
575 580 585
Leu Ser Val Arg Gln Ala Tyr Asp Ile Ala Ala Ser Lys Gln Ile
590 595 600
Leu Ile Pro Asp Val Gln Ala Pro Gly Leu Leu Val Met Val His
605 610 615
Glu Leu Pro Ala Gly Lys Gly Val Gln Leu Thr Ala Leu Asn Phe
620 625 630
Ser Ala Glu Pro Val Ser Glu Thr Ile Cys Leu Pro Gly Val Ala
635 640 645
Pro Gly Pro Val Val Asp Ile Ile His Glu Ser Val Glu Gly Asp
650 655 660
Leu Thr Asp Asn Cys Glu Leu Gln Ile Asn Leu Asp Pro Tyr Glu
665 670 675
Gly Leu Ala Leu Arg Val Val Ser Ala Ala Pro Pro Val Ile
680 685

In addition, the present invention provides a novel trehalose synthase gene with the following nucleotide sequence:

GATCGCTGGC GTACTGCAGG TAGAGCAGGC GCATCGGCCC CCAGGGCGCA TCGGCCGGCT 60
CCGCTGTGCC CTGCTGGTTC ATGAAGCGGA CGAAGCGGCC ATCGCGGAAC CGTGGACGCC 120
ATTCGGGGCT GTCCGGGTCG CGGCTGTCGG TGAGCGTGCG CCACAGGTCG CTGCGAAACG 180
GCGGACCGCT CCAAAGCGCG CCGTGGATGG GATCGCCGAG CAGTTCGTGC AGCTCCCAGG 240
AACGTTGCGA ATGCAGCGCG CCGAGGCTCA GGCCATGCAG ATACAGGCGC GGTCGGCGTT 300
CGGCCGGCAG TTCGGTCCAG TAGCCATAGA TCTCGGCGAA TAGCGCGCGG GCCACGTCGC 360
GGCCGTAGTC GGCCTCCACC AGCAGCGCCA GCGGGCTGTT CAGATAGGAG TACTGCAACG 420
CCACGCTGGC GATATCGCCG TGGTGCAGGT ATTCCACTGC GTTCATCGCC GCCGGGTCGA 480
TCCAGCCGGT ACCGGTGGGC GTCACCAGCA CCAGCACCGA TCGCTCGAAG GCGCCGCTGC 540
GCTGCAGCTC GCGCAAGGCC AGACGCGCCC GCTGGCGCGG GGTCTCTGCC GCGCGCAGAC 600
CGACGTAGAC GCGAATCGGC TCGAGCGCCG AGCGGCCGCT CAAGACGCTG ATATCCGCCG 660
CCGACGGGCC GGAGCCGATG AACTCGCGGC CGGTGCGGCC CAGCTCCTCC CAGCGCAGCA 720
ACGAGGCCCG GCTGCCGCTT TTCAGCGGCG AGGCCGGTGG CGCCGTCTCC GGTTCGATCA 780
GGGCGTCGTA CTGCGCGAAG GATGCGTCCA GCATGCGCAG TGCCCGCGCC GCCAGCACAT 840
CGCTGAGCAG CGACCAGAAC AGCGCCAGCG CCACCAGCAC GCCGATCACG TTGGCCAGGC 900
GCCGTGGCAG CACGCGGTCG GCGTGCCGCG AGACGAAGCG CGACACCAGC CGATACAGAC 960
GCGCCAGCGT CAGCAGGATG AGAAAGGTCG CCAGCGCGGT GAGAATGACT TCGAGCAGGT 1020
GCGCACTGCT CACCGGCGGC ATGCCCATCA GCGCGCGTAC CGCGTTCTGC CAGCCGGCGA 1080
CCTGGCTGAG GAAATACCCG GCCAGCAGCA GGCAGCCGAC CGCGATCAGC AGATTGACCC 1140
GCTCGCGCTG CCAGCCTGGG CGCTCCGGCA GTTCCAGATA GCGCCACAGC CAGCGCCAGA 1200
ACACGCCGAG GCCATAGCCC ACCGCCAGCG CCGCGCCGGC CAGCACGCCC TGGCTCAGCG 1260
TCGAGCGCGG CAGCAGCGAT GGCGTCAGCG CCGCGCAGAA GAACAGCGTG CCCAGCAGCA 1320
GGCCGAAACC GGACAGCGAG CGCCAGATAT AGAGGACGGG CAGGTGCAGC ATGAAGATCT 1380
CCGCGGTCGG GTGACGGCGT CGCGCCTCGG CATATCGAGG CGTGTCCGGT CGTGCGGTTC 1440
CCGTGATGGT CCGCAGCAGG CCAATCCGAT GCAACGATGG CCGAGCGGCC GACTCAAACG 1500
TCTACATTTC CCTAGTGCTG CCGGAACCGA TCGCCG 1536
ATG AGC ATC CCA GAC AAC ACC TAT ATC GAA TGG CTG GTC AGC CAG TCC 1584
ATG CTG CAT GCG GCC CGC GAG CGG TCG CGT CAT TAC GCC GGC CAG GCG 1632
CGT CTC TGG CAG CGG CCT TAT GCC CAG GCC CGC CCG CGC GAT GCC AGC 1680
GCC ATC GCC TCG GTG TGG TTC ACC GCC TAT CCG GCG GCC ATC ATC ACG 1728
CCG GAA GGC GGC ACG GTA CTC GAG GCC CTC GGC GAC GAC CGC CTC TGG 1776

AGT GCG CTC TCC GAA CTC GGC GTG CAG GGC ATC CAC AAC GGG CCG ATG 1824

AAG CGT TCC GGT GGC CTG CGC GGA CGC GAG TTC ACC CCG ACC ATC GAC 1872

GGC AAC TTC GAC CGC ATC AGC TTC GAT ATC GAC CCG AGC CTG GGG ACC 1920

GAG GAG CAG ATG CTG CAG CTC AGC CGG GTG GCC GCG GCG CAC AAC GCC 1968

ATC GTC ATC GAC GAC ATC GTG CCG GCA CAC ACC GGC AAG GGT GCC GAC 2016

TTC CGC CTC GCG GAA ATG GCC TAT GGC GAC TAC CCC GGG CTG TAC CAC 2064

ATG GTG GAA ATC CGC GAG GAG GAC TGG GAG CTG CTG CCC GAG GTG CCG 2112

GCC GGG CGT GAT TCG GTC AAC CTG CTG CCG CCG GTG GTC GAC CGG CTC 2160

AAG GAA AAG CAC TAC ATC GTC GGC CAG CTG CAG CGG GTG ATC TTC TTC 2208

GAG CCG GGC ATC AAG GAC ACC GAC TGG AGC GTC ACC GGC GAG GTC ACC 2256

GGG GTC GAC GGC AAG GTG CGT CGC TGG GTC TAT CTG CAC TAC TTC AAG 2304

GAG GGC CAG CCG TCG CTG AAC TGG CTC GAC CCG ACC TTC GCC GCG CAG 2352

CAG CTG ATC ATC GGC GAT GCG CTG CAC GCC ATC GAC GTC ACC GGC GCC 2400

CGG GTG CTG CGC CTG GAC GCC AAC GGC TTC CTC GGC GTG GAA CGG CGC 2448

GCC GAG GGC ACG GCC TGG TCG GAG GGC CAC CCG CTG TCC GTC ACC GGC 2496

AAC CAG CTG CTC GCC GGG GCG ATC CGC AAG GCC GGC GGC TTC AGC TTC 2544

CAG GAG CTG AAC CTG ACC ATC GAT GAC ATC GCC GCC ATG TCC CAC GGC 2592

GGG GCC GAT CTG TCC TAC GAC TTC ATC ACC CGC CCG GCC TAT CAC CAT 2640

GCG TTG CTC ACC GGC GAT ACC GAA TTC CTG CGC ATG ATG CTG CGC GAA 2688

GTG CAC GCC TTC GGC ATC GAC CCG GCG TCA CTG ATC CAT GCG CTG CAG 2736

AAC CAT GAC GAG TTG ACC CTG GAG CTG GTG CAC TTC TGG ACG CTG CAC 2784

GCC TAC GAC CAT TAC CAC TAC AAG GGC CAG ACC CTG CCC GGC GGC CAC 2832

CTG CGC GAA CAT ATC CGC GAG GAA ATG TAC GAG CGG CTG ACC GGC GAA 2880

CAC GCG CCG TAC AAC CTC AAG TTC GTC ACC AAC GGG GTG TCC TGC ACC 2928

ACC GCC AGC GTG ATC GCC GCG GCG CTT AAC ATC CGT GAT CTG GAC GCC 2976

ATC GGC CCG GCC GAG GTG GAG CAG ATC CAG CGT CTG CAT ATC CTG CTG 3024

GTG ATG TTC AAT GCC ATG CAG CCC GGC GTG TTC GCC CTC TCC GGC TGG 3072

GAT CTG GTC GGC GCC CTG CCG CTG GCG CCC GAG CAG GTC GAG CAC CTG 3120

ATG GGC GAT GGC GAT ACC CGC TGG ATC AAT CGC GGC GGC TAT GAC CTC 3168

GCC GAT CTG GCG CCG GAG GCG TCG GTC TCC GCC GAA GGC CTG CCC AAG 3216

GCC CGC TCG CTG TAC GGC AGC CTG GCC GAG CAG CTG CAG CGG CCA GGC 3264

TCC TTC GCC TGC CAG CTC AAG CGC ATC CTC AGC GTG CGC CAG GCC TAC 3312

GAC ATC GCT GCC AGC AAG CAG ATC CTG ATT CCG GAT GTG CAG GCG CCG 3360

GGA CTC CTG GTG ATG GTC CAC GAG CTG CCT GCC GGC AAG GGC GTG CAG 3408
CTC ACG GCA CTG AAC TTC AGC GCC GAG CCG GTC AGC GAG ACC ATC TGC 3456
CTG CCC GGC GTG GCG CCC GGC CCG GTG GTG GAC ATC ATT CAC GAG AGT 3504
GTG GAG GGC GAC CTC ACC GAC AAC TGC GAG CTG CAG ATC AAC CTC GAC 3552
CCG TAC GAG GGG CTT GCC CTG CGT GTG GTG AGC GCC GCG CCG CCG GTG 3600
ATC TGA GCGC 3610
CCTCTTCGCG CGCCCCGGGT CCGCCGCTAT AGTGCGCAGC GCCTGGGGCG CGCATTGCCC 3670
TCGCCGTCGA GACCAGCCCG TGTCGTTCAC TTCGCTTTTC CGCCTTGCGC TGCTGCCGCT 3730
GGCGCTGCTT GCCGCACCCG TCTGGGCGCA GACCGCCTGC CCGCCCGGCC AGCAGCCGAT 3790
CTGCCTGAGC GGCAGCTGCC TCTGCGTGCC GGCCGCCGCC AGCGATCCAC AGGCGGTCTA 3850
CGACCGCGTG CAGCGTATGG CTACGCTGGC CCTGCAGAAC TGGATCCAGC AGTCGCGCGA 3910
CCGCCTGATG GCCGGCGGCG TCGAGCCGAT ACCGCTGCAC ATCCGCTCGC AGCTCGAGCC 3970
GTATTTCGAT CTTGCCGTGC TGGAGAGTGC GCGGTACCGC GTCGGCGACG AGGTGGTGCT 4030
GACTGCCGGC AACACCCTGC TGCGCAACCC GGACGTCAAT GCCGTGACCC TGATCGACGT 4090
CATCGTCTTC CGCCACGAGG AGGATGCCCG GGACAACGTC GCGCTCTGGG CCCATGAGCT 4150
CAAGCACGTC GAGCAATATC TGGACTGGGG CGTCGCCGAG TTCGCCCGGC GCTATACGCA 4210
GGATTTCCGT GCCGTGGAGC GCCCGGCCTA TGCGCTGGAG CGTGAGGTGG AAGAGGCCCT 4270
GCGCGAGACG CAGACGCGGC GCTGAGCGAG CTGATCGGTG CTGCTGCCCG CACTGGGCTG 4330
AAGCCCACCA ATGACGCCGG CGAAAACGAA AAACCCCGCC GAGGCGGGGT TTCTGACGCG 4390
GGTTGTGCGG TCAGCTCAGA ACGCCGGGAC CACGGCGCCC TTGTACTTTT CCTCGATGAA 4450
CTGGCGTACT TGCTCGCTGT GCAGCGCGGC AGCCAGTTTC TGCATGGCAT CGCTGTCCTT 4510
GTTGTCCGGA CGGGCGACCA GAATGTTCAC GTATGGCGAG TCGCTGCCCT CGATCACCAG 4570
GGCGTCCTGG GTCGGGTTCA GCTTGGCTTC CAGCGCGTAG TTGGTGTTGA TCAGCGCCAG 4630
GTCGACCTGG GTCAGCACGC GCGGCAGAGT CGCGGCTTCC AGTTCGCGGA TCTTGATCTT 4690
CTTCGGGTTC TCGGCGATGT CTTCGGCGTG GCGGTGATGC CGGCGCCGTC CTTCAGACCG 4750
ATC 4753

The present invention also provides a recombinant plasmid containing the trehalose synthase gene with the above nucleotide sequence. In a preferred embodiment, the present invention provides a recombinant plasmid pCJ104 in which the 4.7 kb *Sau*3AI DNA fragment of the trehalose synthase gene of the present invention is cloned into vector plasmid pUC18. This allow for the efficient and high

expression of the trehalose synthase gene. In a more preferred embodiment, the present invention provides a recombinant plasmid pCJ122 in which the 2.5 kb *Bam*HI-*Bgl*II DNA fragment of the trehalose synthase gene of the present invention is included in a vector plasmid pUC18, allowing for a higher expression of the trehalose synthase gene.

The present invention provides a transformed *E. coli* with a recombinant plasmid containing the trehalose synthase gene with the above nucleotide sequence. In a preferred embodiment, the present invention provides a transformed *E. coli* with a recombinant plasmid pCJ104, allowing for production of high levels of the trehalose synthase protein. In a more preferable embodiment, the present invention provides a transformed *E. coli* with the recombinant plasmid pCJ122, allowing for production of even higher levels of the trehalose synthase protein.

The present invention provides a process for producing trehalose which comprises reacting the trehalose synthase protein with the above amino acid sequence with maltose solution to obtain trehalose.

The present invention provides a process for producing trehalose which comprises crushing a transformed *E. coli* with a recombinant plasmid containing the trehalose synthase gene with the above nucleotide sequence and reacting the crushed cells with maltose solution to obtain trehalose. In a preferred embodiment, the present invention provides a process for producing trehalose which comprises crushing a transformed *E. coli* with plasmid pCJ104, centrifuging the crushed cells and reacting the resulting supernatant with maltose solution to obtain trehalose. In a more preferable embodiment, the present invention provides a process for producing trehalose which comprises crushing a transformed *E. coli* with plasmid pCJ122, centrifuging the crushed cells and reacting the resulting supernatant with maltose solution to obtain trehalose.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an analysis of saccharides by thin-layer chromatography to

which a reaction solution containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution was subjected. The symbols G, M and T indicate glucose, maltose and trehalose, respectively.

Figure 2 shows an analysis of saccharides by gas chromatography to which a reaction solution (A) containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution and a standard trehalose specimen (B) were subjected. The symbol Tre indicate trehalose.

Figure 3 shows an analysis of saccharides by high performance liquid chromatography to which a standard trehalose specimen (A), and specimens (B) and (C) were subjected. Specimen (B) was obtained just after a solution containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution was reacted completely. Specimen (C) was obtained by adding trehalase to a reaction solution containing sonicated liquid from *Pseudomonas stutzeri* CJ38 and maltose solution after completion of their reaction. The symbols Tre, Mal and Glu indicate trehalose, maltose and glucose, respectively.

Figure 4 shows a construction map of a recombinant plasmid pCJ104 including a trehalose synthase gene of the present invention.

Figure 5 shows a restriction map of a 4.7 kb *Sau*3AI fragment within a recombinant plasmid pCJ104 of the present invention.

Figure 6 shows a construction map of recombinant plasmids pCJ121 and pCJ122 of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

A microorganism which produces trehalose from maltose by trehalose synthase was isolated from soil and identified as having the morphological and physiological characteristics of *Pseudomonas stutzeri*. *Pseudomonas stutzeri* has not been reported to convert maltose into trehalose. Therefore, the microorganism isolated

by us can be recognized as a novel *Pseudomonas stutzeri* strain and has been designated as *Pseudomonas stutzeri* CJ38.

We constructed the restriction map of a recombinant plasmid pCJ104 of the present invention using various restriction enzymes. Two trehalose synthase gene sequences are known (Biochim. Biophys. Acta 1996, 1290, 1-3 and Biochim. Bophys. Acta 1997, 1334, 28-32). The comparison of the present and known restriction maps revealed that pCJ104 represents different patterns from those known.

Trehalose synthase proteins from known microorganisms have shown similarities in their N-terminus. However, it was found that the N-terminal sequence of the trehalose synthase protein of the present invention is not identical with those of known trehalose synthase proteins. The results are shown in Table 1 below.

Table 1. N-terminal Sequences of Trehalose Synthase Proteins

Source of Trehalose Synthase		N-terminal Sequence
Known Microbes	*Thermus aquaticus* ATCC 33923	M-D-P-L-W-Y-K-D-A-V-I-Y-Q-
	Pimelobacter sp. R48	S-T-V-L-G-E-E-P-E-W-F-R-T-A-V-F-Y-E-
	Pseudomonas putida H262	G-K-W-P-R-P-A-A-F-I-D-
Transformed *E. coli* of the Present Invention		S-I-P-D-N-T-Y-I-E-W-L-V-

The nucleotide sequence of 4.7 kb *Sau*3AI fragment within a recombinant plasmid pCJ104 of the present invention and the amino acid sequence of a trehalose synthase protein expressed therefrom were determined (SEQ ID NO: 1).

In addition, the intact sequence of a trehalose synthase protein of the present invention was compared to those of the trehalose synthase proteins disclosed in Biochim. Biophys. Acta 1996, 1290, 1-3 and Biochim. Biophys. Acta 1997, 1334, 28-

32. The comparison revealed that there are no similarities between them.

The enzymatic conversion reaction was carried out using crushed E. coli transformantsincluding recombinant plasmids pCJ104 or pCJ122. As a result, the titer of trehalose synthase enzyme from the crushed cells of the present invention was considerably higher than that from the wild type *Pseudomonas stutzeri* CJ38.

The properties and availabilities of the plasmids and microorganisms used in and prepared by the present invention are shown in Table 2 below.

Table 2

Microbes and Plasmids	Properties	Availability
Pseudomonas stutzeri CJ38	Wild type strain producing the trehalose synthase enzyme of the present invention	KFCC-10985
E. coli NM522	hsd△5, △(lac⁻pro) [F', Pro⁺, lacI^{q}Z△M15]	Amersham
E. coli ATCC35467	[malP,Q::Tn5 ompBCS1 F⁻ araD139△(argF-lac) 205U169 rpsL150 relA1 flbB5301 deoC1 ptsF25]	ATCC
pCJ104	pUC18 containing 4.7 kb Sau3AI DNA fragment (trehalose synthase gene), Ap^r	Constructed
pCJ121	pUC18 containing 3.35 kb KpnI DNA fragment (trehalose synthase gene), Ap^r	Constructed (Control)
pCJ122	pUC18 containing 2.5 kb BamHI-BglII DNA fragment (trehalose synthase gene), Ap^r	Constructed
pCJ123	pUC18 containing 1.2 kb BamHI-EcoRI DNA fragment	Constructed (Control)
pUC18 and pUC19	Ap^r, 2.7 kb	New England Biolabs

Nutrient medium (0.3% broth, 0.5% peptone, pH 6.8) and LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) were used for cultivation of *Pseudomonas stutzeri* and *E. coli*, respectively. For the culture of cells transformed by electroporation, SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was used. MacConkey agar medium (4% bacto MacConkey agar base, 2.0% maltose, pH 7.0) was used in cloning the trehalose synthase gene. Ampicillin was added in a concentration of 50 mg/L. Gene Pulser (Bio-Rad) was used in transformation of *E. coli* by electroporation. The genetic manipulation used in the present invention was carried out in accordance with procedures described in *Molecular Cloning, Laboratory Manual*, 2nd ed., Sambrook, J., E.F. Frishc and T. Maniatis and *Guide to Molecular Cloning Techniques, Methods in Enzymol.* Vol. 152, Berger, S.L., A.R. Kimme.

The enzymatic reaction is conducted at pH 6.0 to 7.0, preferably pH 7.0 to 10, and at temperatures of 4°C to 45°C, preferably 20°C to 40°C. Maltose can be used as a substrate in a concentration of less than 50%. The trehalose synthase enzyme can be used in a pure form or in crushed cells.

The following examples illustrate the present invention. From the foregoing description and the following examples, it is believed that those skilled in the art would be able to carry out the invention completely.

Example 1

Screening of Microorganism

A platinum loop of microorganisms, isolated from soil, was inoculated in a 500 ml Erlenmeyer flask containing 50 ml of LB culture solution (0.5% of yeast extract, 1.0% of bactotrypton, 0.5% of salt) and cultured at 28°C for 2 days. The culture was centrifuged at 4°C, 8,000 rpm, for 5 minutes. The cells were collected and washed with physiological saline. The washed cells were suspended in 10 ml of phosphate buffer solution (10 mM, pH 7.0). The cells were crushed by an ultrasonicater and the crushed cells were centrifuged at 4°C, 1,200 rpm, for 20 minutes and the supernatant was used as a crude enzymatic solution. The

concentration of the protein in the crude enzymatic solution was determined by the Bredford method. 100 μg of protein was mixed with 20 μl of 100 mM maltose and 10 μl of 100 mM phosphate buffer solution (pH 7.0). Distilled water was added to the mixture until the total volume reached 100 μl and the reaction occurred at 30°C for 20 hours. The saccharides present in the reaction solution were analyzed by TLC, HPLC, and GC.

Example 2

Analysis of Trehalose by Thin-layer Chromatography (Figure 1)

After the reaction was completed, 5 μl of the reaction solution were spotted on Kieselgel 60 TLC (Merck, Germany) and placed in a vessel containing a solvent system of n-butanol-pyridine-water (7:3:1) to develop the specimens. It was sprayed with a solution of 20% sulfuric acid in methyl alcohol and dried at 110°C for 10 minutes. The saccharides in the specimens were thus specified. Among at least 1,000 soil microorganisms investigated, two were confirmed to have the ability to convert maltose into trehalose. Figure 1 shows that trehalose did not exist in the specimens prior to the reaction but, after completion of the reaction, saccharides were detected at the site of a standard trehalose specimen.

Example 3

Analysis of Trehalose by Gas Chromatography (Figure 2)

After completion of the reaction, 10 μl of the reaction solution was dried by a reduced pressure dryer. The dried product was dissolved in 20 μl of dimethylformamide and the resulting solution was mixed with the same volume of bis(trimethyl)trifluoracetamide to form trimethylsilane derivatives. One μl of aliquot was used in GC analysis. As shown in Figure 2, the peak of the reaction solution was observed to occur at the same time as with a standard trehalose specimen.

Example 4

Analysis of Trehalose by High Performance Liquid Chromatography (Figure 3)

After the reaction was completed, half of the reaction solution was mixed with the same volume of phenol to remove proteins. The specimen solution thus obtained was used in the HPLC analysis. The peak of the specimen was observed to occur at the same time as with a standard trehalose specimen. The remaining half of the reaction solution was heated to 100°C for 10 minutes to terminate enzyme activity. It was reacted at 37°C for 10 minutes with trehalase (Sigma) which specifically acts on α-1,1-trehalose. After completion of the reaction, the solution was mixed with the same volume of phenol solution to remove proteins. The solution obtained thus was subjected to HPLC, and as a result the peak disappeared at the same time as with a standard trehalose.

Example 5

Identification of Microorganism Capable of Converting Maltose into Trehalose

The soil microorganism of the present invention was observed by electron microscope and is characterized by rod shaped bacteria with flagellum. It was also characterized as aerobic by an O/F test and by Gram-negative. The physiological characteristics of the microorganism are summarized in Table 1. These characteristics of the present microorganism were compared to those of microorganisms described in *Bergy's Manual of Systemic Bacteriology*, 1984 and in patent publications, and it was classified as *Pseudomonas stutzeri*, because it is almost identical to that microorganism, physiologically and morphologically.

Table 1

DP3 -	OFG +	GC +	ACE -	ESC -	PLI -
URE -	CIT +	MAL +	TDA -	PXB -	LAC -
MLT +	MAN +	XYL -	RAF -	SOR -	SUC -
INO -	ADO -	COU -	H2S -	ONP -	RHA -
ARA -	GLU -	ARG -	LYS -	ORN -	OXI -
TLA -					

Example 6

Cloning of Trehalose Synthase Gene (Figure 4)

(1) Isolation of Chromosomal DNA from *Pseudomonas stutzeri*

Pseudomonas stutzeri was grown in a nutrient medium and at an early resting stage, cells were recovered by centrifugation. The recovered cells were washed twice with TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The washed cells were suspended in 20 mL of STE buffer (20% sucrose, 10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) and 5 mg/mL of lysozyme and RNase A were added to the suspension. The reaction occurred at 37°C for 2 hours. After the reaction was completed, SDS was added up to a concentration of 1% and the reaction continued at 37°C for 30 minutes. This solution was reacted with the same volume of phenol for 4 hours and was subjected to centrifugation. 5M NaCl was added to the resulting supernatant until its concentration reached 0.1 M. Using a glass bar, a two-fold volume of anhydrous ethanol was added to obtain chromosomal DNA. The chromosomal DNA was washed with 70% ethanol and dissolved in TE solution for use in the next experiment.

(2) Preparation of Genomic Library

The pure chromosomal DNAs isolated from *Pseudomonas stutzeri* were partially digested with restriction enzyme *Sau*3AI at 37°C for 15 to 30 minutes. The restriction enzyme was inactivated with heat and agarose gel electrophoresis was carried out to obtain 3 to 10 kb DNA fragments. As shown in Figure 5, plasmid pUC18 was digested with *Bam*HI and was treated with calf intestinal phosphatase. The cleaved DNAs were mixed with 3 to 10 kb DNA fragments previously obtained and ligation with T4 DNA ligase was allowed at 15°C for 16 hours. The recombinants thus obtained were used for transformation. The transformation was carried out by electroporation as follows. *E. coli* NM522 was cultured on LB medium for 14 to 15 hours. The resulting culture was inoculated on 1L LB so that initial absorbency became 0.07 to 0.1 at 600 nm, and then cultivation was allowed until the absorbency reached 0.8. The cells were centrifuged and suspended in 1L of HEPES [N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid)] buffer solution. The cells were again centrifuged and suspended in 500 ml of cold sterile deionized distilled water.

The cells were again centrifuged and suspended in 20 ml of 10% glycerol solution. The cells were again centrifuged and suspended in 2 to 3 ml of 10% glycerol solution so that the cell concentration was adjusted to 2-4 x 1,010/ml. The cell suspension was rapidly frozen and stored at -70°C. The frozen cells could be used for about one month during which time their transformation frequency did not decrease. 40 μL of frozen cell suspension was thawed in ice and the restored suspension was mixed with the ligated DNA solution. The mixture was put in a gene pulser cuvette with a diameter of 0.2 cm and the capacitance and strength of electric field was fixed at 25 uF and 12.5 kV/cm, respectively. After a single electric pulse was passed at resistance of 200 to 400 Ω, 1 ml of SOC medium was immediately added and cultured at 37°C for 1 hour. The culture was streaked on LB-ampicillin agar medium and cultivation was allowed for 24 hours to obtain at least fifty thousand colonies. These colonies were together cultured in LB broth for 2 hours. DNA was purely isolated using an alkaline lysis and the genomic library was constructed therefrom.

(3) Cloning of Trehalose Synthase Gene

E. coli ATCC35467, which is unable to utilize maltose as a carbon source, was transformed with the genomic library obtained from the above by electroporation. The transformed cells were streaked on a MacConkey-ampicillin agar medium containing 20 g/L of maltose. Once the trehalose synthase gene of *Pseudomonas stutzeri* is introduced into *E. coli*, maltose is converted into glucose by the trehalase present in E. coli. As the resulting glucose is metabolized, pH decreases and thereby the color of the colonies on the MacConkey agar medium changes from yellowish to red. This principle was applied to the present cloning system. The transformed *E. coli* ATCC35467 with the genomic library was cultured on a MacConkey agar medium to obtain red colonies. The isolation of plasmid DNA revealed that it contained about 4.7 kb DNA fragment. The plasmid was designated as pCJ104. To assay enzymes, *E. coli* ATCC35467/pUC18 (control), *E. coli* ATCC35467/pCJ104 and wild type *Pseudomonas stutzeri* CJ38 were cultured. *E. coli* cells were grown on a LB medium until their early resting stage. *Pseudomonas stutzeri* CJ38 was grown on a nutrient medium. The cells were separated by centrifugation and crushed. The crushed cells were reacted with 20% maltose as substrate in 20 mM diethanolamine as buffer

solution. at pH of 8.5 to 9.0 and a temperature of 35°C. 1.0% trichloroacetic acid was added to the reaction solution. which was then subjected to centrifugation and high performance liquid chromatography to assay the quantities of maltose and trehalose. The results are shown in Table 3 below.

Table 3. Enzyme Titration

Microorganisms	Non-enzymatic activities (U*/mg of protein)	Culture Titer (U/ml of culture solution)
Pseudomonas stutzeri CJ38	0.1	0.023
E. coli ATCC35467/pUC18	0	0
E. coli ATCC35467/pCJ104	0.26	0.175

*U=μmol trehalose/minutes

Example 7

Restriction Map Construction of Trehalose Synthase Gene (Figure 5)

The plasmid pCJ104 was separated using conventional methods and treated with various restriction enzymes to construct a restriction map.

The plasmid pCJ104 was subjected to single, double, and triple-digest procedures using about twenty restriction enzymes, such as *Aat*II, *Bam*HI, *Eco*RI, *Eco*RV, *Kpn*I, *Nco*I, *Nde*I, *Pst*I, *Sac*I, *Sac*II, *Sal*I, *Sph*I and *Xho*I. DNA fragments were analyzed by electrophoresis through agarose gel and compared to construct the restriction map.

Example 3

Subcloning of Trehalose Synthase Gene and Enzyme Assay

(1) Subcloning of Trehalose Synthase Gene (Figure 6)

A subcloning was carried out to determine the sites of the trehalose synthase gene in 4.7 kb plasmid pCJ104. The plasmid pCJ104 was cleaved with *Kpn*I and a 3.35 kb fragment was isolated. This fragment was introduced into vector pUC18/*Kpn*I/CIP and *E. coli* NM522 was transformed with the resulting recombinant. The recombinant plasmid pCJ121 with a directional cloning of 3.35 kb fragment into pUC18/*Kpn*I was constructed. In addition, the plasmid pCJ104 was cleaved with double digestions of *Bam*HI and *Bgl*II. The 2.5 kb *Bam*HI-*Bgl*II fragment thus obtained was purified and ligated into pUC18/*Bam*HI/CIP, followed by transformation of *E. coli* NM522 with the recombinant. The recombinant plasmid pCJ122 with directional cloning of 2.5 kb *Bam*HI-*Bgl*II fragment into pUC18/*Bam*HI was constructed. Finally, the plasmid pCJ104 was double-digested with *Bam*HI and *Eco*RI and the resulting 1.2 kb *Bam*HI-*Eco*RI fragment was purified. This fragment was ligated into vector pUC18/*Bam*HI/*Eco*RI and *E. coli* NM522 was transformed with the recombinant. The recombinant plasmid pCJ123 was constructed.

E. coli ATCC35467 was transformed with each of the constructed recombinant plasmids. The transformants were cultured on a MacConkey-ampicilline agar medium containing 2.0% maltose (20 g/L) and the color of the colonies formed therefrom was observed. It was observed that the *E. coli* ATCC35467 carrying pCJ121 and pCJ122 formed red colonies but that the *E. coli* ATCC35467 carrying pCJ123 formed yellow colonies since it did not decompose maltose. Therefore, it can be seen that the trehalose synthase gene is located in the larger 2.5 kb *Bam*HI-*Bgl*II fragment, rather than in the 1.2 kb *Bam*HI-*Eco*RI fragment.

(2) Titration of Trehalose Synthase of Transformant Containing Subcloned Plasmid

Transformed *E. coli* ATCC35467/pCJ121, ATCC35467/pCJ122 and ATCC35467/pCJ123 were cultured on an LB-Ap medium until the early resting stage. The cells were recovered by centrifugation and washed twice with an appropriate volume of 20 mM diethanolamine solution. The washed cells were suspended in an

appropriate volume of 20 mM diethanolamine solution and crushed by ultrasonicator. The crushed cells were centrifuged and the supernatant obtained therefrom was used as enzymatic liquid. The supernatant was reacted with 20% maltose solution containing 20 mM diethanolamine, pH 8.5 to 9.0 at 35°C. 1.0% trichloroacetic acid was added to the reaction solution, and centrifugation and HPLC were conducted for analysis. One unit of enzyme activity was defined as a quantity of enzyme when it produced 1 μmol of trehalose per minute. The results are shown in Table 5 below.

According to the double titration, the enzyme titer of *E. coli* ATCC35467/pCJ122 was the highest. *E. coli* ATCC35467/pCJ122 was cultured in high density under the conditions described in Table 6 below in 5 L fermenter. As a result, the non-enzymatic activity was 5.0 U/mg of protein, equal to that obtained by culturing it on an LB medium, and the titer of the trehalose synthase enzyme in the high density culture was increased to 30 U/ml of culture (Table 5). The non-enzymatic activity and culture titer of *E. coli* ATCC35467/pCJ122 were increased 50 times and about 1,300 times, respectively, compared to wild type *Pseudomonas stutzeri*.

Table 5

Microorganisms	Non-enzymatic Activity (U/mg of protein)	Culture Titer of 5 L Fermenter (U/ml of culture)
E. coli ATCC35467/pCJ121	0.43	-
E. coli ATCC35467/pCJ122	4.95	30
E. coli ATCC35467/pCJ123	0	-

Table 6

Fermentation Medium	g/L	Culture Condition
glycerol	50	pH 7.0
$(NH_4)_2SO_4$	6	Temperature of 33°C
KH_2PO_4	2	800 rpm

$MgSO_4 \cdot 7H_2O$	1	1.0 vvm
Yeast Extract	5	
Trace Elements	1 ml	
Amino Acids (Threonine, Leucine, Isoleucine, Valine, Histidine, Arginine)	0.5	

IND

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RAW SEQUENCE LISTING DATE: 03/05/2002
PATENT APPLICATION: **US/09/937,215** TIME: 13:58:25

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and A Process
  6       for Producing Trehalose
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VERIFICATION SUMMARY DATE: 03/05/2002
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ccgtgatggt ccgcagcagg ccaatccgat gcaacgatgg ccgagcggcc gactcaaacg    1500

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                                       Met Ser Ile Pro Asp Asn
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acc tat atc gaa tgg ctg gtc agc cag tcc atg ctg cat gcg gcc cgc      1602
Thr Tyr Ile Glu Trp Leu Val Ser Gln Ser Met Leu His Ala Ala Arg
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gag cgg tcg cgt cat tac gcc ggc cag gcg cgt ctc tgg cag cgg cct      1650
Glu Arg Ser Arg His Tyr Ala Gly Gln Ala Arg Leu Trp Gln Arg Pro
        25                  30                  35

tat gcc cag gcc cgc ccg cgc gat gcc agc gcc atc gcc tcg gtg tgg      1698
Tyr Ala Gln Ala Arg Pro Arg Asp Ala Ser Ala Ile Ala Ser Val Trp
    40                  45                  50

ttc acc gcc tat ccg gcg gcc atc atc acg ccg gaa ggc ggc acg gta      1746
Phe Thr Ala Tyr Pro Ala Ala Ile Ile Thr Pro Glu Gly Gly Thr Val
55                  60                  65                  70

ctc gag gcc ctc ggc gac gac cgc ctc tgg agt gcg ctc tcc gaa ctc      1794
Leu Glu Ala Leu Gly Asp Asp Arg Leu Trp Ser Ala Leu Ser Glu Leu
                75                  80                  85

ggc gtg cag ggc atc cac aac ggg ccg atg aag cgt tcc ggt ggc ctg      1842
Gly Val Gln Gly Ile His Asn Gly Pro Met Lys Arg Ser Gly Gly Leu
            90                  95                  100

cgc gga cgc gag ttc acc ccg acc atc gac ggc aac ttc gac cgc atc      1890
Arg Gly Arg Glu Phe Thr Pro Thr Ile Asp Gly Asn Phe Asp Arg Ile
        105                 110                 115

agc ttc gat atc gac ccg agc ctg ggg acc gag gag cag atg ctg cag      1938
Ser Phe Asp Ile Asp Pro Ser Leu Gly Thr Glu Glu Gln Met Leu Gln
    120                 125                 130
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ctc agc cgg gtg gcc gcg gcg cac aac gcc atc gtc atc gac gac atc     1986
Leu Ser Arg Val Ala Ala Ala His Asn Ala Ile Val Ile Asp Asp Ile
135                 140                 145                 150

gtg ccg gca cac acc ggc aag ggt gcc gac ttc cgc ctc gcg gaa atg     2034
Val Pro Ala His Thr Gly Lys Gly Ala Asp Phe Arg Leu Ala Glu Met
                155                 160                 165

gcc tat ggc gac tac ccc ggg ctg tac cac atg gtg gaa atc cgc gag     2082
Ala Tyr Gly Asp Tyr Pro Gly Leu Tyr His Met Val Glu Ile Arg Glu
            170                 175                 180

gag gac tgg gag ctg ctg ccc gag gtg ccg gcc ggg cgt gat tcg gtc     2130
Glu Asp Trp Glu Leu Leu Pro Glu Val Pro Ala Gly Arg Asp Ser Val
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aac ctg ctg ccg ccg gtg gtc gac cgg ctc aag gaa aag cac tac atc     2178
Asn Leu Leu Pro Pro Val Val Asp Arg Leu Lys Glu Lys His Tyr Ile
    200                 205                 210

gtc ggc cag ctg cag cgg gtg atc ttc ttc gag ccg ggc atc aag gac     2226
Val Gly Gln Leu Gln Arg Val Ile Phe Phe Glu Pro Gly Ile Lys Asp
215                 220                 225                 230

acc gac tgg agc gtc acc ggc gag gtc acc ggg gtc gac ggc aag gtg     2274
Thr Asp Trp Ser Val Thr Gly Glu Val Thr Gly Val Asp Gly Lys Val
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cgt cgc tgg gtc tat ctg cac tac ttc aag gag ggc cag ccg tcg ctg     2322
Arg Arg Trp Val Tyr Leu His Tyr Phe Lys Glu Gly Gln Pro Ser Leu
            250                 255                 260

aac tgg ctc gac ccg acc ttc gcc gcg cag cag ctg atc atc ggc gat     2370
Asn Trp Leu Asp Pro Thr Phe Ala Ala Gln Gln Leu Ile Ile Gly Asp
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gcg ctg cac gcc atc gac gtc acc ggc gcc cgg gtg ctg cgc ctg gac     2418
Ala Leu His Ala Ile Asp Val Thr Gly Ala Arg Val Leu Arg Leu Asp
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gcc aac ggc ttc ctc ggc gtg gaa cgg cgc gcc gag ggc acg gcc tgg     2466
Ala Asn Gly Phe Leu Gly Val Glu Arg Arg Ala Glu Gly Thr Ala Trp
295                 300                 305                 310

tcg gag ggc cac ccg ctg tcc gtc acc ggc aac cag ctg ctc gcc ggg     2514
Ser Glu Gly His Pro Leu Ser Val Thr Gly Asn Gln Leu Leu Ala Gly
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gcg atc cgc aag gcc ggc ggc ttc agc ttc cag gag ctg aac ctg acc     2562
Ala Ile Arg Lys Ala Gly Gly Phe Ser Phe Gln Glu Leu Asn Leu Thr
            330                 335                 340

atc gat gac atc gcc gcc atg tcc cac ggc ggg gcc gat ctg tcc tac     2610
Ile Asp Asp Ile Ala Ala Met Ser His Gly Gly Ala Asp Leu Ser Tyr
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gac ttc atc acc cgc ccg gcc tat cac cat gcg ttg ctc acc ggc gat     2658
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Asp Phe Ile Thr Arg Pro Ala Tyr His His Ala Leu Leu Thr Gly Asp
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acc gaa ttc ctg cgc atg atg ctg cgc gaa gtg cac gcc ttc ggc atc     2706
Thr Glu Phe Leu Arg Met Met Leu Arg Glu Val His Ala Phe Gly Ile
375                 380                 385                 390

gac ccg gcg tca ctg atc cat gcg ctg cag aac cat gac gag ttc acc     2754
Asp Pro Ala Ser Leu Ile His Ala Leu Gln Asn His Asp Glu Phe Thr
                395                 400                 405

ctg gag ctg gtg cac ttc tgg acg ctg cac gcc tac gac cat tac cac     2802
Leu Glu Leu Val His Phe Trp Thr Leu His Ala Tyr Asp His Tyr His
            410                 415                 420

tac aag ggc cag acc ctg ccc ggc ggc cac ctg cgc gaa cat atc cgc     2850
Tyr Lys Gly Gln Thr Leu Pro Gly Gly His Leu Arg Glu His Ile Arg
        425                 430                 435

gag gaa atg tac gag cgg ctg acc ggc gaa cac gcg ccg tac aac ctc     2898
Glu Glu Met Tyr Glu Arg Leu Thr Gly Glu His Ala Pro Tyr Asn Leu
    440                 445                 450

aag ttc gtc acc aac ggg gtg tcc tgc acc acc gcc agc gtg atc gcc     2946
Lys Phe Val Thr Asn Gly Val Ser Cys Thr Thr Ala Ser Val Ile Ala
455                 460                 465                 470

gcg gcg ctt aac atc cgt gat ctg gac gcc atc ggc ccg gcc gag gtg     2994
Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala Ile Gly Pro Ala Glu Val
                475                 480                 485

gag cag atc cag cgt ctg cat atc ctg ctg gtg atg ttc aat gcc atg     3042
Glu Gln Ile Gln Arg Leu His Ile Leu Leu Val Met Phe Asn Ala Met
            490                 495                 500

cag ccc ggc gtg ttc gcc ctc tcc ggc tgg gat ctg gtc ggc gcc ctg     3090
Gln Pro Gly Val Phe Ala Leu Ser Gly Trp Asp Leu Val Gly Ala Leu
        505                 510                 515

ccg ctg gcg ccc gag cag gtc gag cac ctg atg ggc gat ggc gat acc     3138
Pro Leu Ala Pro Glu Gln Val Glu His Leu Met Gly Asp Gly Asp Thr
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cgc tgg atc aat cgc ggc ggc tat gac ctc gcc gat ctg gcg ccg gag     3186
Arg Trp Ile Asn Arg Gly Gly Tyr Asp Leu Ala Asp Leu Ala Pro Glu
535                 540                 545                 550

gcg tcg gtc tcc gcc gaa ggc ctg ccc aag gcc cgc tcg ctg tac ggc     3234
Ala Ser Val Ser Ala Glu Gly Leu Pro Lys Ala Arg Ser Leu Tyr Gly
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agc ctg gcc gag cag ctg cag cgg cca ggc tcc ttc gcc tgc cag ctc     3282
Ser Leu Ala Glu Gln Leu Gln Arg Pro Gly Ser Phe Ala Cys Gln Leu
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Lys Arg Ile Leu Ser Val Arg Gln Ala Tyr Asp Ile Ala Ala Ser Lys
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        585                 590                 595

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cac gag ctg cct gcc ggc aag ggc gtg cag ctc acg gca ctg aac ttc     3426
His Glu Leu Pro Ala Gly Lys Gly Val Gln Leu Thr Ala Leu Asn Phe
615                 620                 625                 630

agc gcc gag ccg gtc agc gag acc atc tgc ctg ccc ggc gtg gcg ccc     3474
Ser Ala Glu Pro Val Ser Glu Thr Ile Cys Leu Pro Gly Val Ala Pro
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ggc ccg gtg gtg gac atc att cac gag agt gtg gag ggc gac ctc acc     3522
Gly Pro Val Val Asp Ile Ile His Glu Ser Val Glu Gly Asp Leu Thr
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Leu Arg Val Val Ser Ala Ala Pro Pro Val Ile
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Ala Ile Ala Ser Val Trp Phe Thr Ala Tyr Pro Ala Ala Ile Ile Thr
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Lys Arg Ser Gly Gly Leu Arg Gly Arg Glu Phe Thr Pro Thr Ile Asp
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Phe Arg Leu Ala Glu Met Ala Tyr Gly Asp Tyr Pro Gly Leu Tyr His
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Met Val Glu Ile Arg Glu Glu Asp Trp Glu Leu Leu Pro Glu Val Pro
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Ala Gly Arg Asp Ser Val Asn Leu Leu Pro Pro Val Val Asp Arg Leu
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Lys Glu Lys His Tyr Ile Val Gly Gln Leu Gln Arg Val Ile Phe Phe
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Glu Pro Gly Ile Lys Asp Thr Asp Trp Ser Val Thr Gly Glu Val Thr
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Gly Val Asp Gly Lys Val Arg Arg Trp Val Tyr Leu His Tyr Phe Lys
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Val His Ala Phe Gly Ile Asp Pro Ala Ser Leu Ile His Ala Leu Gln
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        435                 440                 445

His Ala Pro Tyr Asn Leu Lys Phe Val Thr Asn Gly Val Ser Cys Thr
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Thr Ala Ser Val Ile Ala Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala
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Ile Gly Pro Ala Glu Val Glu Gln Ile Gln Arg Leu His Ile Leu Leu
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Val Met Phe Asn Ala Met Gln Pro Gly Val Phe Ala Leu Ser Gly Trp
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Asp Leu Val Gly Ala Leu Pro Leu Ala Pro Glu Gln Val Glu His Leu
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Ala Arg Ser Leu Tyr Gly Ser Leu Ala Glu Gln Leu Gln Arg Pro Gly
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Ser Phe Ala Cys Gln Leu Lys Arg Ile Leu Ser Val Arg Gln Ala Tyr
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Asp Ile Ala Ala Ser Lys Gln Ile Leu Ile Pro Asp Val Gln Ala Pro
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<400> 6

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1 5 10

SEQUENCE LISTING

GENERAL INFORMATION:

APPLICANT
NAME: Cheil Jedang Corporation
STREET:
CITY: Seoul
COUNTRY: Republic of Korea
POSTAL CODE (ZIP): 100-095
TELEPHONE: 82 2 7268 286
TELEFAX: 82 2 7268 219
TELEX:

TITLE OF INVENTION: Trehalose Synthase Protein, Gene, Plasmids, Microorganisms, and A Process for Producing Trehalose

NUMBER OF SEQUENCES: 1

CORRESPONDENCE ADDRESS:
ADDRESS: 500, 5-ga, Namdaemun-ro, Chung-ku
STREET:
CITY: Seoul
STATE OR PROVINCE:
COUNTRY: Republic of Korea
POSTAL CODE: 100-095

COMPUTER READABLE FORM:
MEDIUM TYPE : Floppy disk
COMPUTER: IBM PC Compatible
OPERATING SYSTEM: Windows 95
SOFTWARE: Notepad, Hangul 97

CURRENT APPLICATION DATA:
APPLICATION NUMBER:
FILING DATE:
CLASSIFICATION:

PRIOR APPLICATION DATA:
COUNTRY: Republic of Korea
APPLICATION NUMBER:

FILING DATE:
CLASSIFICATION:

ATTORNEY/AGENT INFORMATION:
NAME: Choi, Hak Hyun and Hwang, Ju Myung
REGISTRATION NUMBER:
REFERENCE/DOCKET NUMBER:

TELECOMMUNICATION INFORMATION:
TELEPHONE: 82 2 365 2727
TELEFAX: 82 2 365 3370
ELECTRONIC MAIL: patent@hmpj.com

INFORMATION FOR SEQ ID NO: 1

SEQUENCE CHARACTERISTICS:
LENGTH: 4753
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear

MOLECULE TYPE: Trehalose Synthase Gene

HYPOTHETICAL:

ANTI-SENSE:

ORIGINAL SOURCE:
ORGANISM: Pseudomonas stutzeri
STRAIN: CJ38

SEQUENCE DESCRIPTION: SEQ ID NO: 1

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Pro Glu Gly Gly Thr Val Leu Glu Ala Leu Gly Asp Asp Arg Leu Trp

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Ser Ala Leu Ser Glu Leu Gly Val Gln Gly Ile His Asn Gly Pro Met

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Ala Leu Leu Thr Gly Asp Thr Glu Phe Leu Arg Met Met Leu Arg Glu

5.

GTG CAC GCC TTC GGC ATC GAC CCG GCG TCA CTG ATC CAT GCG CTG CAG 2736
Val His Ala Phe Gly Ile Asp Pro Ala Ser Leu Ile His Ala Leu Gln

AAC CAT GAC GAG TTG ACC CTG GAG CTG GTG CAC TTC TGG ACG CTG CAC 2784
Asn His Asp Glu Leu Thr Leu Glu Leu Val His Phe Trp Thr Leu His

GCC TAC GAC CAT TAC CAC TAC AAG GGC CAG ACC CTG CCC GGC GGC CAC 2832
Ala Tyr Asp His Tyr His Tyr Lys Gly Gln Thr Leu Pro Gly Gly His

CTG CGC GAA CAT ATC CGC GAG GAA ATG TAC GAG CGG CTG ACC GGC GAA 2880
Leu Arg Glu His Ile Arg Glu Glu Met Tyr Glu Arg Leu Thr Gly Glu

CAC GCG CCG TAC AAC CTC AAG TTC GTC ACC AAC GGG GTG TCC TGC ACC 2928
His Ala Pro Tyr Asn Leu Lys Phe Val Thr Asn Gly Val Ser Cys Thr

ACC GCC AGC GTG ATC GCC GCG GCG CTT AAC ATC CGT GAT CTG GAC GCC 2976
Thr Ala Ser Val Ile Ala Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala

ATC GGC CCG GCC GAG GTG GAG CAG ATC CAG CGT CTG CAT ATC CTG CTG 3024
Ile Gly Pro Ala Glu Val Glu Gln Ile Gln Arg Leu His Ile Leu Leu

GTG ATG TTC AAT GCC ATG CAG CCC GGC GTG TTC GCC CTC TCC GGC TGG 3072
Val Met Phe Asn Ala Met Gln Pro Gly Val Phe Ala Leu Ser Gly Trp

GAT CTG GTC GGC GCC CTG CCG CTG GCG CCC GAG CAG GTC GAG CAC CTG 3120
Asp Leu Val Gly Ala Leu Pro Leu Ala Pro Glu Gln Val Glu His Leu

ATG GGC GAT GGC GAT ACC CGC TGG ATC AAT CGC GGC GGC TAT GAC CTC 3168
Met Gly Asp Gly Asp Thr Arg Trp Ile Asn Arg Gly Gly Tyr Asp Leu

GCC GAT CTG GCG CCG GAG GCG TCG GTC TCC GCC GAA GGC CTG CCC AAG 3216
Ala Asp Leu Ala Pro Glu Ala Ser Val Ser Ala Glu Gly Leu Pro Lys

GCC CGC TCG CTG TAC GGC AGC CTG GCC GAG CAG CTG CAG CGG CCA GGC 3264
Ala Arg Ser Leu Tyr Gly Ser Leu Ala Glu Gln Leu Gln Arg Pro Gly

TCC TTC GCC TGC CAG CTC AAG CGC ATC CTC AGC GTG CGC CAG GCC TAC 3312
Ser Phe Ala Cys Gln Leu Lys Arg Ile Leu Ser Val Arg Gln Ala Tyr

GAC ATC GCT GCC AGC AAG CAG ATC CTG ATT CCG GAT GTG CAG GCG CCG 3360
Asp Ile Ala Ala Ser Lys Gln Ile Leu Ile Pro Asp Val Gln Ala Pro

GGA CTC CTG GTG ATG GTC CAC GAG CTG CCT GCC GGC AAG GGC GTG CAG 3408
Gly Leu Leu Val Met Val His Glu Leu Pro Ala Gly Lys Gly Val Gln

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CTC ACG GCA CTG AAC TTC AGC GCC GAG CCG GTC AGC GAG ACC ATC TGC      3456
Leu Thr Ala Leu Asn Phe Ser Ala Glu Pro Val Ser Glu Thr Ile Cys

CTG CCC GGC GTG GCG CCC GGC CCG GTG GTG GAC ATC ATT CAC GAG AGT      3504
Leu Pro Gly Val Ala Pro Gly Pro Val Val Asp Ile Ile His Glu Ser

GTG GAG GGC GAC CTC ACC GAC AAC TGC GAG CTG CAG ATC AAC CTC GAC      3552
Val Glu Gly Asp Leu Thr Asp Asn Cys Glu Leu Gln Ile Asn Leu Asp

CCG TAC GAG GGG CTT GCC CTG CGT GTG GTG AGC GCC GCG CCG CCG GTG      3600
Pro Tyr Glu Gly Leu Ala Leu Arg Val Val Ser Ala Ala Pro Pro Val

ATC TGA GCGC                                                        3610
Ile

CCTCTTCGCG CGCCCCGGGT CCGCCGCTAT AGTGCGCAGC GCCTGGGGCG CGCATTGCCC   3670
TCGCCGTCGA GACCAGCCCG TGTCGTTCAC TTCGCTTTTC CGCCTTGCGC TGCTGCCGCT   3730
GGCGCTGCTT GCCGCACCCG TCTGGGCGCA GACCGCCTGC CCGCCCGGCC AGCAGCCGAT   3790
CTGCCTGAGC GGCAGCTGCC TCTGCGTGCC GGCCGCCGCC AGCGATCCAC AGGCGGTCTA   3850
CGACCGCGTG CAGCGTATGG CTACGCTGGC CCTGCAGAAC TGGATCCAGC AGTCGCGCGA   3910
CCGCCTGATG GCCGGCGGCG TCGAGCCGAT ACCGCTGCAC ATCCGCTCGC AGCTCGAGCC   3970
GTATTTCGAT CTTGCCGTGC TGGAGAGTGC GCGGTACCGC GTCGGCGACG AGGTGGTGCT   4030
GACTGCCGGC AACACCCTGC TGCGCAACCC GGACGTCAAT GCCGTGACCC TGATCGACGT   4090
CATCGTCTTC CGCCACGAGG AGGATGCCCG GGACAACGTC GCGCTCTGGG CCCATGAGCT   4150
CAAGCACGTC GAGCAATATC TGGACTGGGG CGTCGCCGAG TTCGCCCGGC GCTATACGCA   4210
GGATTTCCGT GCCGTGGAGC GCCCGGCCTA TGCGCTGGAG CGTGAGGTGG AAGAGGCCCT   4270
GCGCGAGACG CAGACGCGGC GCTGAGCGAG CTGATCGGTG CTGCTGCCCG CACTGGGCTG   4330
AAGCCCACCA ATGACGCCGG CGAAAACGAA AAACCCCGCC GAGGCGGGGT TTCTGACGCG   4390
GGTTGTGCGG TCAGCTCAGA ACGCCGGGAC CACGGCGCCC TTGTACTTTT CCTCGATGAA   4450
CTGGCGTACT TGCTCGCTGT GCAGCGCGGC AGCCAGTTTC TGCATGGCAT CGCTGTCCTT   4510
GTTGTCCGGA CGGGCGACCA GAATGTTCAC GTATGGCGAG TCGCTGCCCT CGATCACCAG   4570
GGCGTCCTGG GTCGGGTTCA GCTTGGCTTC CAGCGCGTAG TTGGTGTTGA TCAGCGCCAG   4630
GTCGACCTGG GTCAGCACGC GCGGCAGAGT CGCGGCTTCC AGTTCGCGGA TCTTGATCTT   4690
CTTCGGGTTC TCGGCGATGT CTTCGGCGTG GCGGTGATGC CGGCGCCGTC CTTCAGACCG   4750
ATC                                                                 4753
```

SEQUENCE LISTING

GENERAL INFORMATION:

APPLICANT
NAME: Cheil Jedang Corporation
STREET:
CITY: Seoul
COUNTRY: Republic of Korea
POSTAL CODE (ZIP): 100-095
TELEPHONE: 82 2 7268 286
TELEFAX: 82 2 7268 219
TELEX:

TITLE OF INVENTION: Trehalose Synthase Protein, Gene, Plasmids, Microorganisms, and A Process for Producing Trehalose

NUMBER OF SEQUENCES: 1

CORRESPONDENCE ADDRESS:
ADDRESS: 500, 5-ga, Namdaemun-ro, Chung-ku
STREET:
CITY: Seoul
STATE OR PROVINCE:
COUNTRY: Republic of Korea
POSTAL CODE: 100-095

COMPUTER READABLE FORM:
MEDIUM TYPE : Floppy disk
COMPUTER: IBM PC Compatible
OPERATING SYSTEM: Windows 95
SOFTWARE: Notepad, Hangul 97

CURRENT APPLICATION DATA:
APPLICATION NUMBER:
FILING DATE:
CLASSIFICATION:

PRIOR APPLICATION DATA:
COUNTRY: Republic of Korea
APPLICATION NUMBER:

FILING DATE:
CLASSIFICATION:

ATTORNEY/AGENT INFORMATION:
NAME: Choi, Hak Hyun and Hwang, Ju Myung
REGISTRATION NUMBER:
REFERENCE/DOCKET NUMBER:

TELECOMMUNICATION INFORMATION:
TELEPHONE: 82 2 365 2727
TELEFAX: 82 2 365 3370
ELECTRONIC MAIL: patent@hmpj.com

INFORMATION FOR SEQ ID NO: 1

SEQUENCE CHARACTERISTICS:
LENGTH: 4753
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear

MOLECULE TYPE: Trehalose Synthase Gene

HYPOTHETICAL:

ANTI-SENSE:

ORIGINAL SOURCE:
ORGANISM: Pseudomonas stutzeri
STRAIN: CJ38

SEQUENCE DESCRIPTION: SEQ ID NO: 1

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GATCGCTGGC GTACTGCAGG TAGAGCAGGC GCATCGGCCC CCAGGGCGCA TCGGCCGGCT     60
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GCGGACCGCT CCAAAGCGCG CCGTGGATGG GATCGCCGAG CAGTTCGTGC AGCTCCCAGG    240
AACGTTGCGA ATGCAGCGCG CCGAGGCTCA GGCCATGCAG ATACAGGCGC GGTCGGCGTT    300
CGGCCGGCAG TTCGGTCCAG TAGCCATAGA TCTCGGCGAA TAGCGCGCGG GCCACGTCGC    360
GGCCGTAGTC GGCCTCCACC AGCAGCGCCA GCGGGCTGTT CAGATAGGAG TACTGCAACG    420
CCACGCTGGC GATATCGCCG TGGTGCAGGT ATTCCACTGC GTTCATCGCC GCCGGGTCGA    480
TCCAGCCGGT ACCGGTGGGC GTCACCAGCA CCAGCACCGA TCGCTCGAAG GCGCCGCTGC    540
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GCTGCAGCTC GCGCAAGGCC AGACGCGCCC GCTGGCGCGG GGTCTCTGCC GCGCGCAGAC 600
CGACGTAGAC GCGAATCGGC TCGAGCGCCG AGCGGCCGCT CAAGACGCTG ATATCCGCCG 660
CCGACGGGCC GGAGCCGATG AACTCGCGGC CGGTGCGGCC CAGCTCCTCC CAGCGCAGCA 720
ACGAGGCCCG GCTGCCGCTT TTCAGCGGCG AGGCCGGTGG CGCCGTCTCC GGTTCGATCA 780
GGGCGTCGTA CTGCGCGAAG GATGCGTCCA GCATGCGCAG TGCCCGCGCC GCCAGCACAT 840
CGCTGAGCAG CGACCAGAAC AGCGCCAGCG CCACCAGCAC GCCGATCACG TTGGCCAGGC 900
GCCGTGGCAG CACGCGGTCG GCGTGCCGCG AGACGAAGCG CGACACCAGC CGATACAGAC 960
GCGCCAGCGT CAGCAGGATG AGAAAGGTCG CCAGCGCGGT GAGAATGACT TCGAGCAGGT 1020
GCGCACTGCT CACCGGCGGC ATGCCCATCA GCGCGCGTAC CGCGTTCTGC CAGCCGGCGA 1080
CCTGGCTGAG GAAATACCCG GCCAGCAGCA GGCAGCCGAC CGCGATCAGC AGATTGACCC 1140
GCTCGCGCTG CCAGCCTGGG CGCTCCGGCA GTTCCAGATA GCGCCACAGC CAGCGCCAGA 1200
ACACGCCGAG GCCATAGCCC ACCGCCAGCG CCGCGCCGGC CAGCACGCCC TGGCTCAGCG 1260
TCGAGCGCGG CAGCAGCGAT GGCGTCAGCG CCGCGCAGAA GAACAGCGTG CCCAGCAGCA 1320
GGCCGAAACC GGACAGCGAG CGCCAGATAT AGAGGACGGG CAGGTGCAGC ATGAAGATCT 1380
CCGCGGTCGG GTGACGGCGT CGCGCCTCGG CATATCGAGG CGTGTCCGGT CGTGCGGTTC 1440
CCGTGATGGT CCGCAGCAGG CCAATCCGAT GCAACGATGG CCGAGCGGCC GACTCAAACG 1500
TCTACATTTC CCTAGTGCTG CCGGAACCGA TCGCCG 1536

ATG AGC ATC CCA GAC AAC ACC TAT ATC GAA TGG CTG GTC AGC CAG TCC 1584
Met Ser Ile Pro Asp Asn Thr Tyr Ile Glu Trp Leu Val Ser Gln Ser

ATG CTG CAT GCG GCC CGC GAG CGG TCG CGT CAT TAC GCC GGC CAG GCG 1632
Met Leu His Ala Ala Arg Glu Arg Ser Arg His Tyr Ala Gly Gln Ala

CGT CTC TGG CAG CGG CCT TAT GCC CAG GCC CGC CCG CGC GAT GCC AGC 1680
Arg Leu Trp Gln Arg Pro Try Ala Gln Ala Arg Pro Arg Asp Ala Ser

GCC ATC GCC TCG GTG TGG TTC ACC GCC TAT CCG GCG GCC ATC ATC ACG 1728
Ala Ile Ala Ser Val Trp Phe Thr Ala Tyr Pro Ala Ala Ile Ile Thr

CCG GAA GGC GGC ACG GTA CTC GAG GCC CTC GGC GAC GAC CGC CTC TGG 1776
Pro Glu Gly Gly Thr Val Leu Glu Ala Leu Gly Asp Asp Arg Leu Trp

AGT GCG CTC TCC GAA CTC GGC GTG CAG GGC ATC CAC AAC GGG CCG ATG 1824
Ser Ala Leu Ser Glu Leu Gly Val Gln Gly Ile His Asn Gly Pro Met

AAG CGT TCC GGT GGC CTG CGC GGA CGC GAG TTC ACC CCG ACC ATC GAC 1872
Lys Arg Ser Gly Gly Leu Arg Gly Arg Glu Phe Thr Pro Thr Ile Asp

GGC AAC TTC GAC CGC ATC AGC TTC GAT ATC GAC CCG AGC CTG GGG ACC 1920
Gly Asn Phe Asp Arg Ile Ser Phe Asp Ile Asp Pro Ser Leu Gly Thr

GAG GAG CAG ATG CTG CAG CTC AGC CGG GTG GCC GCG GCG CAC AAC GCC 1968
Glu Glu Gln Met Leu Gln Leu Ser Arg Val Ala Ala Ala His Asn Ala

ATC GTC ATC GAC GAC ATC GTG CCG GCA CAC ACC GGC AAG GGT GCC GAC 2016
Ile Val Ile Asp Asp Ile Val Pro Ala His Thr Gly Lys Gly Ala Asp

TTC CGC CTC GCG GAA ATG GCC TAT GGC GAC TAC CCC GGG CTG TAC CAC 2064
Phe Arg Leu Ala Glu Met Ala Tyr Gly Asp Tyr Pro Gly Leu Tyr His

ATG GTG GAA ATC CGC GAG GAG GAC TGG GAG CTG CTG CCC GAG GTG CCG 2112
Met Val Glu Ile Arg Glu Glu Asp Trp Glu Leu Leu Pro Glu Val Pro

GCC GGG CGT GAT TCG GTC AAC CTG CTG CCG CCG GTG GTC GAC CGG CTC 2160
Ala Gly Arg Asp Ser Val Asn Leu Leu Pro Pro Val Val Asp Arg Leu

AAG GAA AAG CAC TAC ATC GTC GGC CAG CTG CAG CGG GTG ATC TTC TTC 2208
Lys Glu Lys His Tyr Ile Val Gly Gln Leu Gln Arg Val Ile Phe Phe

GAG CCG GGC ATC AAG GAC ACC GAC TGG AGC GTC ACC GGC GAG GTC ACC 2256
Glu Pro Gly Ile Lys Asp Thr Asp Trp Ser Val Thr Gly Glu Val Thr

GGG GTC GAC GGC AAG GTG CGT CGC TGG GTC TAT CTG CAC TAC TTC AAG 2304
Gly Val Asp Gly Lys Val Arg Arg Trp Val Tyr Leu His Tyr Phe Lys

GAG GGC CAG CCG TCG CTG AAC TGG CTC GAC CCG ACC TTC GCC GCG CAG 2352
Glu Gly Gln Pro Ser Leu Asn Trp Leu Asp Pro Thr Phe Ala Ala Gln

CAG CTG ATC ATC GGC GAT GCG CTG CAC GCC ATC GAC GTC ACC GGC GCC 2400
Gln Leu Ile Ile Gly Asp Ala Leu His Ala Ile Asp Val Thr Gly Ala

CGG GTG CTG CGC CTG GAC GCC AAC GGC TTC CTC GGC GTG GAA CGG CGC 2448
Arg Val Leu Arg Leu Asp Ala Asn Gly Phe Leu Gly Val Glu Arg Arg

GCC GAG GGC ACG GCC TGG TCG GAG GGC CAC CCG CTG TCC GTC ACC GGC 2496
Ala Glu Gly Thr Ala Trp Ser Glu Gly His Pro Leu Ser Val Thr Gly

AAC CAG CTG CTC GCC GGG GCG ATC CGC AAG GCC GGC GGC TTC AGC TTC 2544
Asn Gln Leu Leu Ala Gly Ala Ile Arg Lys Ala Gly Gly Phe Ser Phe

CAG GAG CTG AAC CTG ACC ATC GAT GAC ATC GCC GCC ATG TCC CAC GGC 2592
Gln Glu Leu Asn Leu Thr Ile Asp Asp Ile Ala Ala Met Ser His Gly

GGG GCC GAT CTG TCC TAC GAC TTC ATC ACC CGC CCG GCC TAT CAC CAT 2640
Gly Ala Asp Leu Ser Tyr Asp Phe Ile Thr Arg Pro Ala Tyr His His

GCG TTG CTC ACC GGC GAT ACC GAA TTC CTG CGC ATG ATG CTG CGC GAA 2688
Ala Leu Leu Thr Gly Asp Thr Glu Phe Leu Arg Met Met Leu Arg Glu

GTG CAC GCC TTC GGC ATC GAC CCG GCG TCA CTG ATC CAT GCG CTG CAG 2736
Val His Ala Phe Gly Ile Asp Pro Ala Ser Leu Ile His Ala Leu Gln

AAC CAT GAC GAG TTG ACC CTG GAG CTG GTG CAC TTC TGG ACG CTG CAC 2784
Asn His Asp Glu Leu Thr Leu Glu Leu Val His Phe Trp Thr Leu His

GCC TAC GAC CAT TAC CAC TAC AAG GGC CAG ACC CTG CCC GGC GGC CAC 2832
Ala Tyr Asp His Tyr His Tyr Lys Gly Gln Thr Leu Pro Gly Gly His

CTG CGC GAA CAT ATC CGC GAG GAA ATG TAC GAG CGG CTG ACC GGC GAA 2880
Leu Arg Glu His Ile Arg Glu Glu Met Tyr Glu Arg Leu Thr Gly Glu

CAC GCG CCG TAC AAC CTC AAG TTC GTC ACC AAC GGG GTG TCC TGC ACC 2928
His Ala Pro Tyr Asn Leu Lys Phe Val Thr Asn Gly Val Ser Cys Thr

ACC GCC AGC GTG ATC GCC GCG GCG CTT AAC ATC CGT GAT CTG GAC GCC 2976
Thr Ala Ser Val Ile Ala Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala

ATC GGC CCG GCC GAG GTG GAG CAG ATC CAG CGT CTG CAT ATC CTG CTG 3024
Ile Gly Pro Ala Glu Val Glu Gln Ile Gln Arg Leu His Ile Leu Leu

GTG ATG TTC AAT GCC ATG CAG CCC GGC GTG TTC GCC CTC TCC GGC TGG 3072
Val Met Phe Asn Ala Met Gln Pro Gly Val Phe Ala Leu Ser Gly Trp

GAT CTG GTC GGC GCC CTG CCG CTG GCG CCC GAG CAG GTC GAG CAC CTG 3120
Asp Leu Val Gly Ala Leu Pro Leu Ala Pro Glu Gln Val Glu His Leu

ATG GGC GAT GGC GAT ACC CGC TGG ATC AAT CGC GGC GGC TAT GAC CTC 3168
Met Gly Asp Gly Asp Thr Arg Trp Ile Asn Arg Gly Gly Tyr Asp Leu

GCC GAT CTG GCG CCG GAG GCG TCG GTC TCC GCC GAA GGC CTG CCC AAG 3216
Ala Asp Leu Ala Pro Glu Ala Ser Val Ser Ala Glu Gly Leu Pro Lys

GCC CGC TCG CTG TAC GGC AGC CTG GCC GAG CAG CTG CAG CGG CCA GGC 3264
Ala Arg Ser Leu Tyr Gly Ser Leu Ala Glu Gln Leu Gln Arg Pro Gly

TCC TTC GCC TGC CAG CTC AAG CGC ATC CTC AGC GTG CGC CAG GCC TAC 3312
Ser Phe Ala Cys Gln Leu Lys Arg Ile Leu Ser Val Arg Gln Ala Tyr

GAC ATC GCT GCC AGC AAG CAG ATC CTG ATT CCG GAT GTG CAG GCG CCG 3360
Asp Ile Ala Ala Ser Lys Gln Ile Leu Ile Pro Asp Val Gln Ala Pro

GGA CTC CTG GTG ATG GTC CAC GAG CTG CCT GCC GGC AAG GGC GTG CAG 3408
Gly Leu Leu Val Met Val His Glu Leu Pro Ala Gly Lys Gly Val Gln

CTC ACG GCA CTG AAC TTC AGC GCC GAG CCG GTC AGC GAG ACC ATC TGC 3456
Leu Thr Ala Leu Asn Phe Ser Ala Glu Pro Val Ser Glu Thr Ile Cys

CTG CCC GGC GTG GCG CCC GGC CCG GTG GTG GAC ATC ATT CAC GAG AGT 3504
Leu Pro Gly Val Ala Pro Gly Pro Val Val Asp Ile Ile His Glu Ser

GTG GAG GGC GAC CTC ACC GAC AAC TGC GAG CTG CAG ATC AAC CTC GAC 3552
Val Glu Gly Asp Leu Thr Asp Asn Cys Glu Leu Gln Ile Asn Leu Asp

CCG TAC GAG GGG CTT GCC CTG CGT GTG GTG AGC GCC GCG CCG CCG GTG 3600
Pro Tyr Glu Gly Leu Ala Leu Arg Val Val Ser Ala Ala Pro Pro Val

ATC TGA GCGC 3610
Ile

CCTCTTCGCG CGCCCCGGGT CCGCCGCTAT AGTGCGCAGC GCCTGGGGCG CGCATTGCCC 3670
TCGCCGTCGA GACCAGCCCG TGTCGTTCAC TTCGCTTTTC CGCCTTGCGC TGCTGCCGCT 3730
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GACTGCCGGC AACACCCTGC TGCGCAACCC GGACGTCAAT GCCGTGACCC TGATCGACGT 4090
CATCGTCTTC CGCCACGAGG AGGATGCCCG GGACAACGTC GCGCTCTGGG CCCATGAGCT 4150
CAAGCACGTC GAGCAATATC TGGACTGGGG CGTCGCCGAG TTCGCCCGGC GCTATACGCA 4210
GGATTTCCGT GCCGTGGAGC GCCCGGCCTA TGCGCTGGAG CGTGAGGTGG AAGAGGCCCT 4270
GCGCGAGACG CAGACGCGGC GCTGAGCGAG CTGATCGGTG CTGCTGCCCG CACTGGGCTG 4330
AAGCCCACCA ATGACGCCGG CGAAAACGAA AAACCCCGCC GAGGCGGGGT TTCTGACGCG 4390
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CTGGCGTACT TGCTCGCTGT GCAGCGCGGC AGCCAGTTTC TGCATGGCAT CGCTGTCCTT 4510
GTTGTCCGGA CGGGCGACCA GAATGTTCAC GTATGGCGAG TCGCTGCCCT CGATCACCAG 4570
GGCGTCCTGG GTCGGGTTCA GCTTGGCTTC CAGCGCGTAG TTGGTGTTGA TCAGCGCCAG 4630
GTCGACCTGG GTCAGCACGC GCGGCAGAGT CGCGGCTTCC AGTTCGCGGA TCTTGATCTT 4690
CTTCGGGTTC TCGGCGATGT CTTCGGCGTG GCGGTGATGC CGGCGCCGTC CTTCAGACCG 4750
ATC 4753

WHAT IS CLAIMED IS:

1. A trehalose synthase protein with the following amino acid sequence:

```
Met Ser Ile Pro Asp Asn Thr Tyr Ile Glu Trp Leu Val Ser Gln
                5                  10                  15
Ser Met Leu His Ala Ala Arg Glu Arg Ser Arg His Tyr Ala Gly
            20                  25                  30
Gln Ala Arg Leu Trp Gln Arg Pro Try Ala Gln Ala Arg Pro Arg
            35                  40                  45
Asp Ala Ser Ala Ile Ala Ser Val Trp Phe Thr Ala Tyr Pro Ala
            50                  55                  60
Ala Ile Ile Thr Pro Glu Gly Gly Thr Val Leu Glu Ala Leu Gly
            65                  70                  75
Asp Asp Arg Leu Trp Ser Ala Leu Ser Glu Leu Gly Val Gln Gly
            80                  85                  90
Ile His Asn Gly Pro Met Lys Arg Ser Gly Gly Leu Arg Gly Arg
            95                  100                 105
Glu Phe Thr Pro Thr Ile Asp Gly Asn Phe Asp Arg Ile Ser Phe
            110                 115                 120
Asp Ile Asp Pro Ser Leu Gly Thr Glu Glu Gln Met Leu Gln Leu
            125                 130                 135
Ser Arg Val Ala Ala Ala His Asn Ala Ile Val Ile Asp Asp Ile
            140                 145                 150
Val Pro Ala His Thr Gly Lys Gly Ala Asp Phe Arg Leu Ala Glu
            155                 160                 165
Met Ala Tyr Gly Asp Tyr Pro Gly Leu Tyr His Met Val Glu Ile
            170                 175                 180
Arg Glu Glu Asp Trp Glu Leu Leu Pro Glu Val Pro Ala Gly Arg
            185                 190                 195
Asp Ser Val Asn Leu Leu Pro Pro Val Val Asp Arg Leu Lys Glu
            200                 205                 210
Lys His Tyr Ile Val Gly Gln Leu Gln Arg Val Ile Phe Phe Glu
            215                 220                 225
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Pro Gly Ile Lys Asp Thr Asp Trp Ser Val Thr Gly Glu Val Thr
230 235 240
Gly Val Asp Gly Lys Val Arg Arg Trp Val Tyr Leu His Tyr Phe
245 250 255
Lys Glu Gly Gln Pro Ser Leu Asn Trp Leu Asp Pro Thr Phe Ala
260 265 270
Ala Gln Gln Leu Ile Ile Gly Asp Ala Leu His Ala Ile Asp Val
275 280 285
Thr Gly Ala Arg Val Leu Arg Leu Asp Ala Asn Gly Phe Leu Gly
290 295 300
Val Glu Arg Arg Ala Glu Gly Thr Ala Trp Ser Glu Gly His Pro
305 310 315
Leu Ser Val Thr Gly Asn Gln Leu Leu Ala Gly Ala Ile Arg Lys
320 325 330
Ala Gly Gly Phe Ser Phe Gln Glu Leu Asn Leu Thr Ile Asp Asp
335 340 345
Ile Ala Ala Met Ser His Gly Gly Ala Asp Leu Ser Tyr Asp Phe
350 355 360
Ile Thr Arg Pro Ala Tyr His His Ala Leu Leu Thr Gly Asp Thr
365 370 375
Glu Phe Leu Arg Met Met Leu Arg Glu Val His Ala Phe Gly Ile
380 385 390
Asp Pro Ala Ser Leu Ile His Ala Leu Gln Asn His Asp Glu Leu
395 400 405
Thr Leu Glu Leu Val His Phe Trp Thr Leu His Ala Tyr Asp His
410 415 420
Tyr His Tyr Lys Gly Gln Thr Leu Pro Gly Gly His Leu Arg Glu
425 430 435
His Ile Arg Glu Glu Met Tyr Glu Arg Leu Thr Gly Glu His Ala
440 445 450
Pro Tyr Asn Leu Lys Phe Val Thr Asn Gly Val Ser Cys Thr Thr
455 460 465

Ala Ser Val Ile Ala Ala Ala Leu Asn Ile Arg Asp Leu Asp Ala
470 475 480
Ile Gly Pro Ala Glu Val Glu Gln Ile Gln Arg Leu His Ile Leu
485 490 495
Leu Val Met Phe Asn Ala Met Gln Pro Gly Val Phe Ala Leu Ser
500 505 510
Gly Trp Asp Leu Val Gly Ala Leu Pro Leu Ala Pro Glu Gln Val
515 520 525
Glu His Leu Met Gly Asp Gly Asp Thr Arg Trp Ile Asn Arg Gly
530 535 540
Gly Tyr Asp Leu Ala Asp Leu Ala Pro Glu Ala Ser Val Ser Ala
545 550 555
Glu Gly Leu Pro Lys Ala Arg Ser Leu Tyr Gly Ser Leu Ala Glu
560 565 570
Gln Leu Gln Arg Pro Gly Ser Phe Ala Cys Gln Leu Lys Arg Ile
575 580 585
Leu Ser Val Arg Gln Ala Tyr Asp Ile Ala Ala Ser Lys Gln Ile
590 595 600
Leu Ile Pro Asp Val Gln Ala Pro Gly Leu Leu Val Met Val His
605 610 615
Glu Leu Pro Ala Gly Lys Gly Val Gln Leu Thr Ala Leu Asn Phe
620 625 630
Ser Ala Glu Pro Val Ser Glu Thr Ile Cys Leu Pro Gly Val Ala
635 640 645
Pro Gly Pro Val Val Asp Ile Ile His Glu Ser Val Glu Gly Asp
650 655 660
Leu Thr Asp Asn Cys Glu Leu Gln Ile Asn Leu Asp Pro Tyr Glu
665 670 675
Gly Leu Ala Leu Arg Val Val Ser Ala Ala Pro Pro Val Ile
680 685

2. A trehalose synthase gene with the following nucleotide sequence:

GATCGCTGGC GTACTGCAGG TAGAGCAGGC GCATCGGCCC CCAGGGCGCA TCGGCCGGCT 60
CCGCTGTGCC CTGCTGGTTC ATGAAGCGGA CGAAGCGGCC ATCGCGGAAC CGTGGACGCC 120
ATTCGGGGCT GTCCGGGTCG CGGCTGTCGG TGAGCGTGCG CCACAGGTCG CTGCGAAACG 180
GCGGACCGCT CCAAAGCGCG CCGTGGATGG GATCGCCGAG CAGTTCGTGC AGCTCCCAGG 240
AACGTTGCGA ATGCAGCGCG CCGAGGCTCA GGCCATGCAG ATACAGGCGC GGTCGGCGTT 300
CGGCCGGCAG TTCGGTCCAG TAGCCATAGA TCTCGGCGAA TAGCGCGCGG GCCACGTCGC 360
GGCCGTAGTC GGCCTCCACC AGCAGCGCCA GCGGGCTGTT CAGATAGGAG TACTGCAACG 420
CCACGCTGGC GATATCGCCG TGGTGCAGGT ATTCCACTGC GTTCATCGCC GCCGGGTCGA 480
TCCAGCCGGT ACCGGTGGGC GTCACCAGCA CCAGCACCGA TCGCTCGAAG GCGCCGCTGC 540
GCTGCAGCTC GCGCAAGGCC AGACGCGCCC GCTGGCGCGG GGTCTCTGCC GCGCGCAGAC 600
CGACGTAGAC GCGAATCGGC TCGAGCGCCG AGCGGCCGCT CAAGACGCTG ATATCCGCCG 660
CCGACGGGCC GGAGCCGATG AACTCGCGGC CGGTGCGGCC CAGCTCCTCC CAGCGCAGCA 720
ACGAGGCCCG GCTGCCGCTT TTCAGCGGCG AGGCCGGTGG CGCCGTCTCC GGTTCGATCA 780
GGGCGTCGTA CTGCGCGAAG GATGCGTCCA GCATGCGCAG TGCCCGCGCC GCCAGCACAT 840
CGCTGAGCAG CGACCAGAAC AGCGCCAGCG CCACCAGCAC GCCGATCACG TTGGCCAGGC 900
GCCGTGGCAG CACGCGGTCG GCGTGCCGCG AGACGAAGCG CGACACCAGC CGATACAGAC 960
GCGCCAGCGT CAGCAGGATG AGAAAGGTCG CCAGCGCGGT GAGAATGACT TCGAGCAGGT 1020
GCGCACTGCT CACCGGCGGC ATGCCCATCA GCGCGCGTAC CGCGTTCTGC CAGCCGGCGA 1080
CCTGGCTGAG GAAATACCCG GCCAGCAGCA GGCAGCCGAC CGCGATCAGC AGATTGACCC 1140
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ACACGCCGAG GCCATAGCCC ACCGCCAGCG CCGCGCCGGC CAGCACGCCC TGGCTCAGCG 1260
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ATC 4753

3. A recombinant plasmid containing the trehalose synthase gene of claim 1.

4. The recombinant plasmid according to claim 1 which is recombinant plasmid pCJ122.

5. A transformed E. coli with the recombinant plasmid of claim 1.

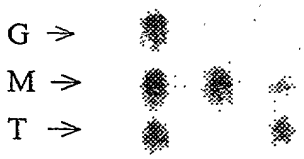
6. The transformant according to claim 5 in which the recombinant plasmid is pCJ122.

7. A process for producing trehalose which comprises reacting the trehalose synthase enzyme of claim 1 with maltose solution to obtain trehalose.

8. A process for producing trehalose which comprises crushing the transformed E. coli of claim 5, centrifuging the crushed bacteria, and reacting the resulting supernatant with maltose solution to obtain trehalose.

9. A novel microorganism *Pseudomonas stutzeri* CJ38 that produces trehalose from maltose.

Fig. 1



C CJ38 CJ38
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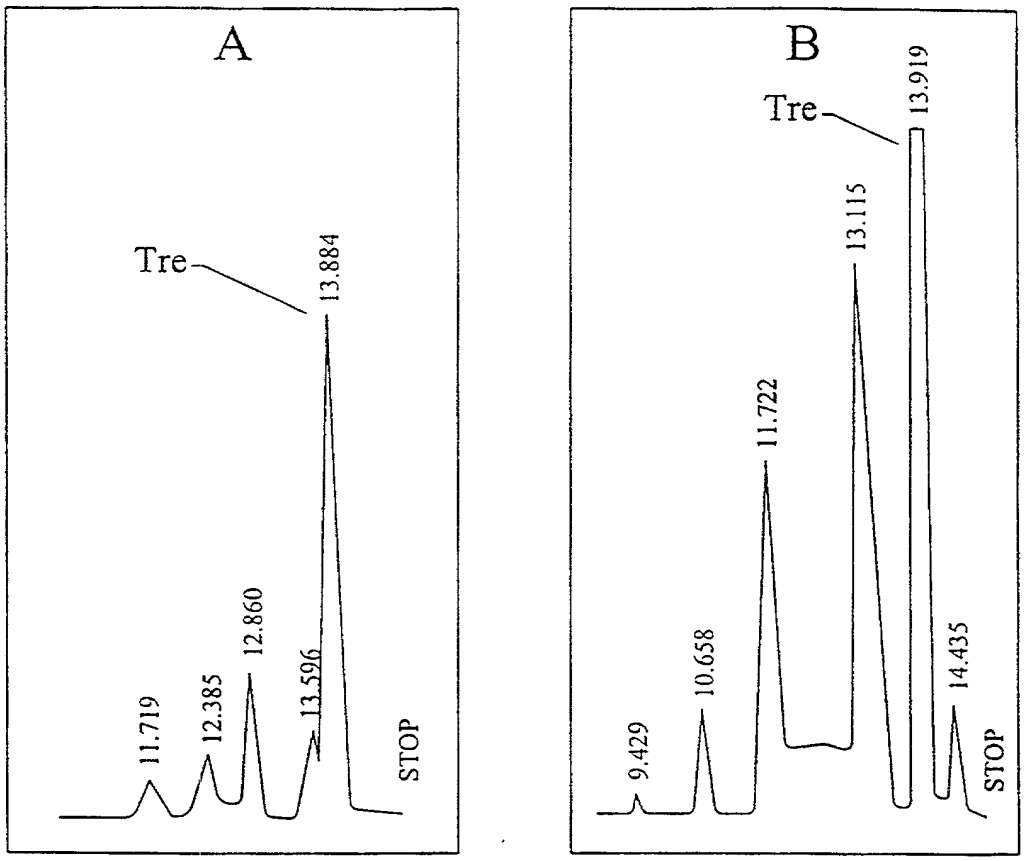
Fig. 2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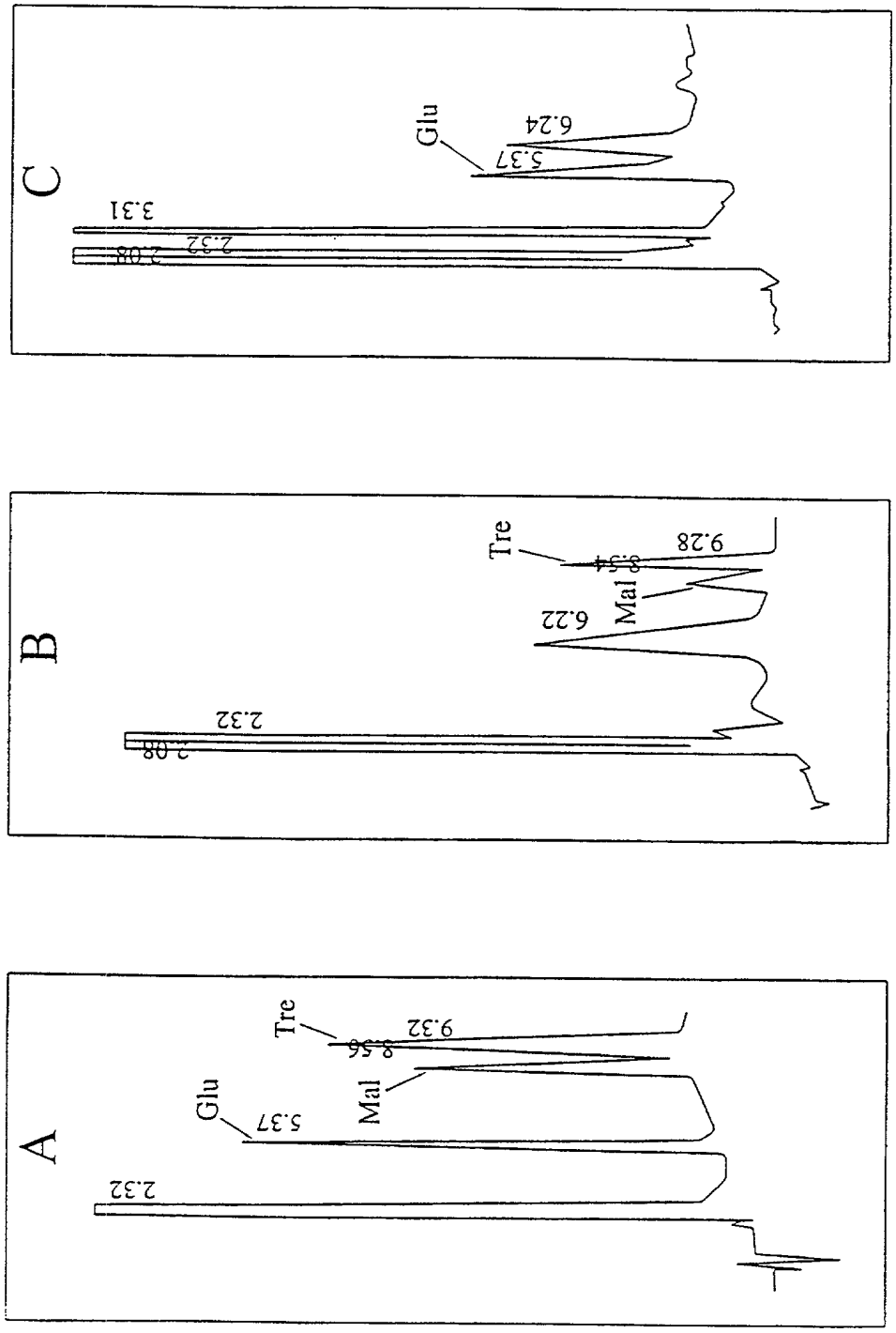
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Fig. 3

Fig. 4

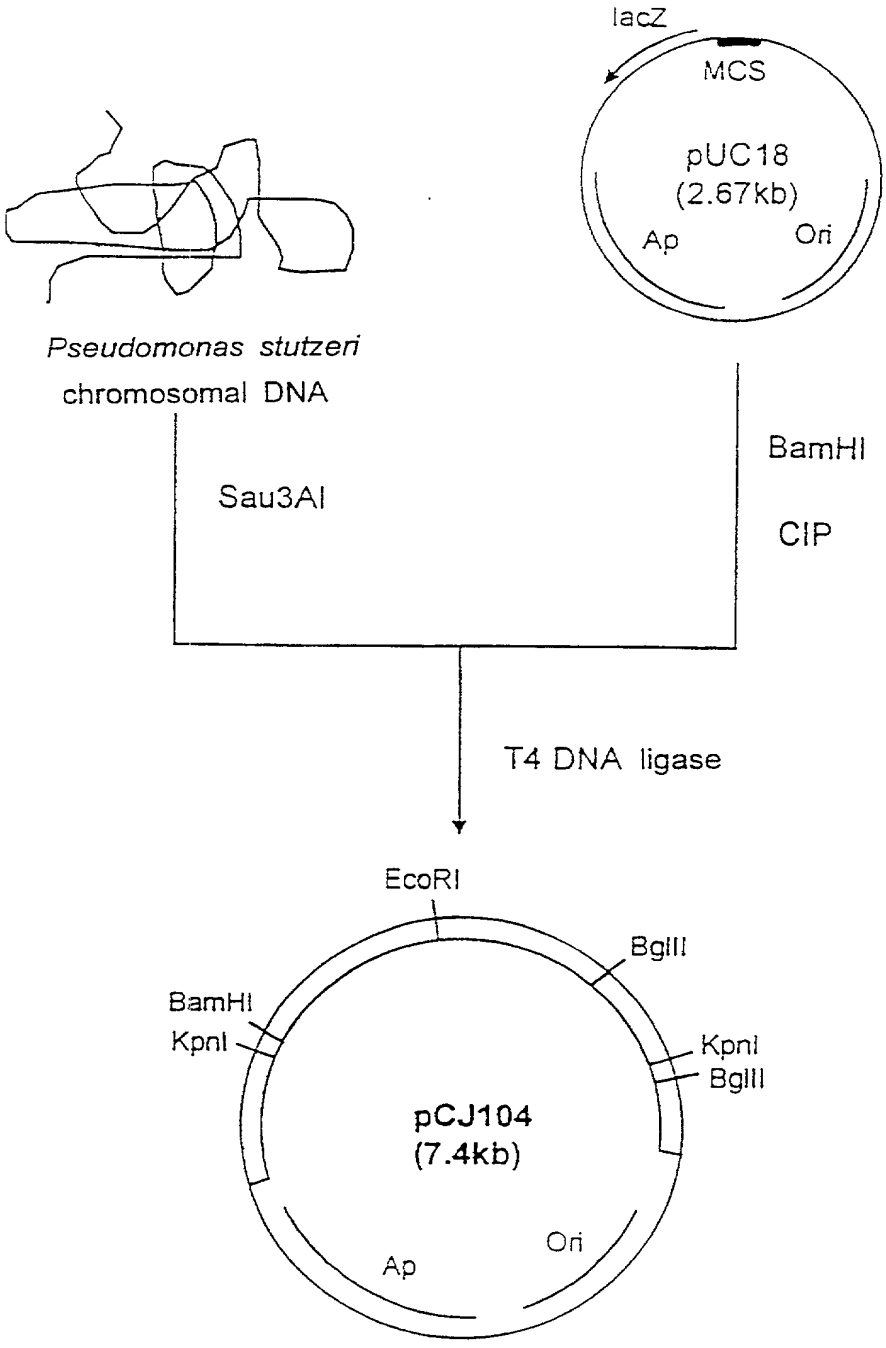


Fig. 5

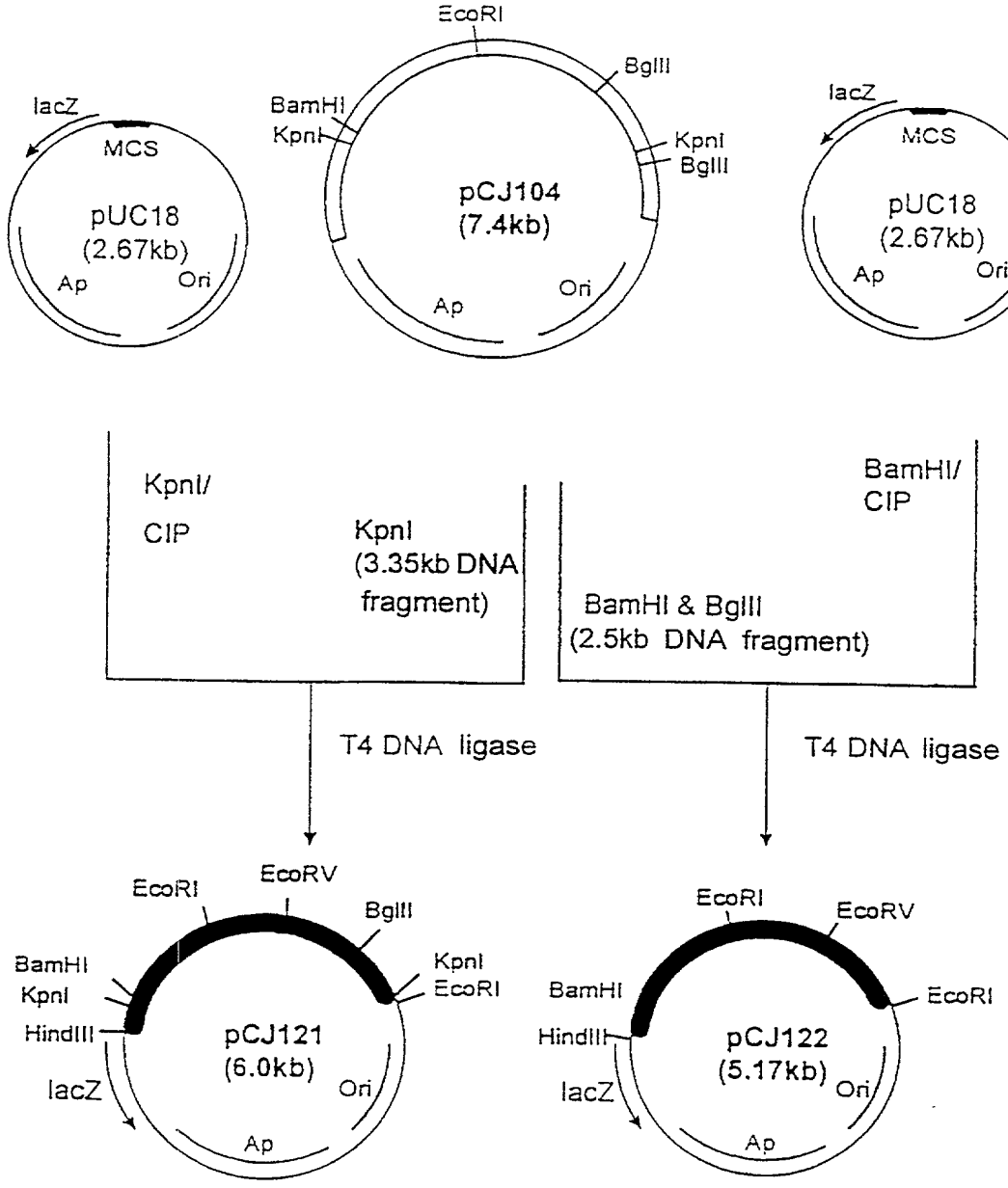
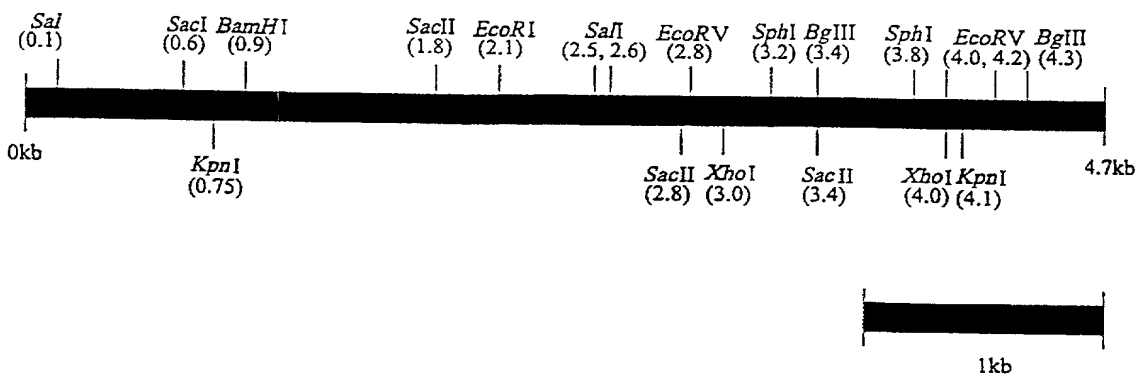


Fig.6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1kb

Attorney Docket No
1599-0206P

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As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title

"TREHALOSE SYNTHASE PROTEIN, GENE, PLASMIDS, MICROORGANISMS, AND A PROCESS FOR PRODUCING TREHAL

Fill in Appropriate Information - For Use Without Specification Attached

the specification of which is attached hereto. If not attached hereto,
the specification was filed on _____ as
United States Application Number _____,
and amended on _____ (if applicable) and/or
the specification was filed on March 24, 1999 as PCT
International Application Number PCT/KR99/00131, and was
amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

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I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representative or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

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Insert Priority Information (if appropriate)

Prior Foreign Application(s)			Priority Claimed	
(Number)	(Country)	(Month/Day/Year Filed)	▯ Yes	▯ No
(Number)	(Country)	(Month/Day/Year Filed)	▯ Yes	▯ No
(Number)	(Country)	(Month/Day/Year Filed)	▯ Yes	▯ No
(Number)	(Country)	(Month/Day/Year Filed)	▯ Yes	▯ No

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Insert Provisional Application(s) (if any)

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More than 12 Months (6 Months for Designs) Prior to the Filing Date of This Application:

Insert Requested Information: (if appropriate)

Country	Application Number	Date of Filing (Month/Day/Year)

I hereby claim the benefit under Title 35, United States Code, ▯ 20 of any United States and/or PCT application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States and/or PCT application in the manner provided by the first paragraph of Title 35, United States Code, ▯ 12, I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, ▯ .56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Insert Prior U.S. Application(s) (if any)

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)
(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)

1599-0206P

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I hereby appoint the practitioners at **CUSTOMER NO. 2292** as my attorneys or agents to prosecute this application and/or an international application based on this application and to transact all business in the United States Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the practitioners, unless the inventor(s) or assignee provides said practitioners with a written notice to the contrary:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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3 OR	Yearn Hung PARK	Yearn Hung Park	Sep. 13, 2001
	Residence (City, State & Country): Nowon-ku, Seoul, Korea	CITIZENSHIP: KRX Republic of Korea	
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4 OR	Sang Ho KWON	Sang Ho Kwon	Sep. 13, 2001
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5 OR	Kwang Ho LEE	Kwang Ho Lee	Sep. 17, 2001
	Residence (City, State & Country): Dongjak-ku, Seoul, Korea	CITIZENSHIP: Republic of Korea KRX	
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1599-0206P

Attorney Docket No.

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MAILING ADDRESS (Complete Street Address including City, State & Country)		

Full Name of Twelfth Inventor, if any see above

GIVEN NAME/FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
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MAILING ADDRESS (Complete Street Address including City, State & Country)		

Full Name of Thirteenth Inventor, if any see above

GIVEN NAME/FAMILY NAME	INVENTOR'S SIGNATURE	DATE*
Residence (City, State & Country)		CITIZENSHIP
MAILING ADDRESS (Complete Street Address including City, State & Country)		

*DATE OF SIGNATURE